1979

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Date

ANTIBODY BINDING AND THE CELL CYCLE:
A STUDY OF THEIR RELATIONSHIP
IN A MOUSE MACROPHAGE-LIKE CELL LINE

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A Thesis Submitted
to the Yale University School of Medicine
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Medicine

1979

ACKNOWLEDGEMENTS

I wish to thank Dr. Janos Varga for his very generous support, encouragement, and teaching during all phases of this project. I am also indebted to Drs. Frank Richards, Robert Rosenstein, William T. Barnes, Mark Horowitz, and David Burkholder and to Valerie Vishno for their help at many times with my work.

CONTENTS

GENERAL INTRODUCTION

A central issue in cell biology is the relationship between a cell's function and its developmental stage in the cell cycle. New techniques for determining cell cycle stage as well as other cell characteristics -- particularly at the macromolecular level of organization -- have provided powerful tools to probe this developmental relationship. In some systems one can now describe the role of the cell cycle in the regulation of cellular events, and demonstrate the interaction between cell cycle and surface membrane events in the control of essential cellular functions. The present paper will tell how we investigated the relationship between the cell cycle and the display of Fc receptors on the surface membrane of a mouse macrophage-like cell line.

The Cell Cycle

The animal cell cycle consists of four phases, demarcated by the periods of DNA synthesis and mitosis: they are S, the phase of chromosomal DNA duplication (6-8 hrs.); G2, a "gap" before division (2-6 hrs.); M, mitosis (1 hr.); and G1, a "gap" between the birth of the new cell and the beginning of S. There is also a hypothetical quiescent stage called G_o, which is a "side-track" off of G1[1]. Control of growth

in general appears to reside in G1; evidence for this is, one, that cells which do not divide for long periods, such as normal hepatocytes, usually remain in G1 or G_o [1]; two, that experimentally-induced changes in the growth rates of cell cultures result from changes in the duration of G1 [2,3,4]; and three, that there appears to be a "restriction point" within G1 at which a cell "decides" either to grow and progress through the remainder of the cell cycle or else to remain quiescent, depending on the availability of nutrients, the cell culture density, the presence of drugs or hormones, and other factors [5].

Cell cycle phases have a significant relationship to cell growth and functioning. Far from following a continuous line of development, cell growth and functioning are often discontinuous, and certain events are specifically associated with certain phases of the cell cycle. Porter et al. [11], studying Chinese Hamster Ovary cells with the scanning electron microscope, found that the numbers of surface blebs, microvilli and ruffles changed as the cells progressed through the cell cycle. Cells in the G_o state, according to Pardee [10], show depleted endoplasmic cisternae, loss of polysomes, increased microfilaments, enzyme changes and changes in transport. Buell et al. [79] found in a rat basophilic leukemia cell line that the development

of basophilic granules depended on growth of the culture into a high-density stationary phase during which the cells have a G1 DNA content (although the G1 state is not in itself sufficient to cause granule development). Investigators have found associations between cell cycle phases and functions as diverse as dexamethasone-induced production of an enzyme in hepatoma cells[12], cell sensitivity to viral infection[13], and production of immunoglobulines by human lymphoid cells[14]. Clearly, the cell cycle is intimately connected with the timing of many kinds of cellular events.

This research project was designed to examine the relationship between the cell cycle and the display of certain surface membrane receptors. There is literature on three possible kinds of relationship: (1) how cell growth or cell cycle changes may affect the display of surface membrane receptors; (2) how receptor-related events may affect cell growth or the cell cycle; and (3) how the cell cycle and receptor display may interact in the regulation of cellular events.

On the first point, cell cycle affecting receptor display, let us review some of the studies that have demonstrated the display of receptors being limited to a certain growth period or cell cycle phase. Fox et al.[16] showed

that normal 3T3 cells contain a receptor site for wheat-germ agglutinin which can be exposed by trypsin treatment at any time in the cell cycle; however, the cells spontaneously display these receptors (i.e., unmask them) only during mitosis. Isersky et al. [18], in a careful study that used several different techniques for looking at the association between cell cycle phases and receptor display, in rat basophilic leukemia cells, found that the number of receptors for IgE rose during G1 only; moreover, it did so while cell volume remained constant -- thereby indicating a true cell cycle association as distinguished from a nonspecific growth-related increase in the quantity of membrane proteins. Finally, not only receptor density but also distribution has been related to the cell cycle: Garrido [19], looking at Chinese Hamster Ovary cells for the distribution of Concanavalin A and wheat germ agglutinin labels, found that there was a greater amount of discontinuity during mitosis than during interphase; he concluded that there is more cell surface receptor clustering in the former cell cycle phase. (This last study, incidentally, is one of the few in which a cell cycle-associated phenomenon was examined by microscopy.)

Some investigators who have not tested specifically for cell cycle phase associations have found associations

between receptor display and growth conditions nevertheless. Krug et al.[20] showed that, whereas unstimulated human lymphocytes have no insulin receptors on their cell surfaces, lymphocytes which are stimulated by the plant mitogen Concanavalin A do develop such receptors. Treatment of both types of cells with phospholipase demonstrated that the effect is due to synthesis of the receptors and not merely to their unmasking. Hoffmann and Kolodny[67] found in 3T3 cells that insulin receptor number per cell was lower in growing fibroblasts and increased as cells entered the stationary phase. Dientsman et al.[32] looked at mouse peritoneal macrophages harvested after starch injection; treatment of the cells in vitro with macrophage growth factor caused these normally resting cells to reinitiate the cell cycle, to develop a "unique iodinatable surface aggregate that was dithiothreitol sensitive," and to become able to bind significantly more IgM than non-macrophage growth factor-treated cells. If we bear in mind that cells in fast-growing, or exponential, cultures are distributed throughout the cell cycle, whereas cells in stationary cultures reside predominantly in G1 or G_o, then we can interpret these experimental results as possibly showing an association with cell cycle stages, rather than with "growth"

or "blast-transformation" in a general sense only.

A good example of such research, in which an unlooked-for cell cycle association might possibly explain a phenomenon that was described only statistically for a whole population, is that of Rhodes[21], who looked at mouse peritoneal macrophages' avidities for IgG. He found that normal (unstimulated) macrophages were heterogeneous in cellular avidity for IgG, as judged by their binding of erythrocytes coated with increasing densities of specific IgG. Rhodes obtained a sigmoid curve, showing a normal or logistic distribution of avidities. Peritoneal exudate macrophages, on the other hand, showed a six-fold increase in the proportion of high-avidity cells. In order to determine whether the change in avidity was confined to a subclass of cells, Rhodes maintained normal peritoneal macrophages in culture for 96 hours and assessed their rosette-forming behavior: little change was detectable for the first 24 hours, but thereafter the number of rosette-forming cells increased progressively to almost 90%, demonstrating that virtually all cells were capable of being activated. Rhodes concluded that these findings demonstrated "receptor activation" of macrophages, such as might occur with proteases released by activated macrophages which would act on surface moieties to increase

receptor accessibility.

If, for the sake of argument, we assume the truth of the hypothesis that the number of displayed Fc receptors changes as a function of cell cycle stage, then we can interpret Rhodes's findings in a different way. Since the in vitro increase in the number of high-avidity cells was achieved upon culture in fresh serum, it would seem likely that this change has a relationship to the typical response of cell cultures to fresh serum, namely a re-initiation of progress through the cell cycle. The presence of a lag phase after introduction of the serum is a part of this response[5,68,69]. Second, Rhodes refers to a paper by Walker[66], who reports that different groups of rabbit peritoneal exudate cells, separated according to cell mass by centrifugation in Ficoll solution, correlated with differences in antigen- and antibody-binding capacity similar to those that Rhodes found. On the basis of his in vitro study Rhodes disputes Walker's assertion that the groups which were separated by centrifugation constitute subclasses that are functionally different; he suggests instead that the differences may be due to the older, activated residents of the peritoneal cavity having ingested more mineral oil. We, on the other hand, favor explaining the difference as a function of differences between cell cycle stages among members of the population; indeed,

other investigators have used centrifugation specifically to separate groups of cells by cell cycle stage[18,30], and that particular correlation has been very thoroughly established[77]. If one takes this approach then Walker's work essentially confirms our findings below. Finally, Rhodes published a study[59] demonstrating that the normal increase in the number of macrophages forming rosettes, in culture, was inhibited by insulin and by cAMP but augmented by cGMP. It is very possible that these substances might operate through an effect on cell cycle progression; and it is only necessary to point out that both cAMP, cGMP[1,68,4] and insulin[64] have been shown to affect the growth of cells in culture. In sum, we can plausibly view the "normal" population of peritoneal macrophages as a "resting" population with most members in the same cell cycle phase (and displaying a probabilistically "normal" range of avidities); and the "activation" process as a signal to the cell to move to a new stage of the cell cycle, not only to prepare for division but also to enable a larger number of Fc receptors to be displayed.

The same kind of interpretation can be applied at least in part to Walker's other findings about differences between macrophage populations which have been separated by centrifugation. He found that rabbit peritoneal cells differed both in the subcellular localization of ingested antigen

(labelled Bovine Serum Albumin) and in the levels of phagocytic activity: the smaller cells had greater phagocytic activity, and localized the ingested antigen in a lysosomal compartment where it was rapidly degraded; larger cells, at the bottom of the centrifugation tube, had lower phagocytic activity and localized the ingested antigen in a perinuclear, probably "storage," compartment with a low rate of degradation[41]. The finding that phagocytic activity correlates negatively with Fc receptor display is surprising and merits further investigation. Walker has also found heterogeneity for the production of immunogenic RNA species, among groups of macrophages separated by centrifugation[70]. Walker's hypothesis is that this separation procedure identifies true functional subclasses; this idea he supports by demonstrating that there are functional differences among macrophages found at different sites in the body and also among those within the same tissue (such as spleen and lymph nodes). Of course this latter finding[60] does not establish that there is true heterogeneity, or "subclass" distinction, among cells from peritoneal fluid separated by centrifugation. It may very well be that the differences can be explained by cell cycle stage differences. One way to test this hypothesis would be to take Walker's separated population groups,

grow them independently and see whether their ranges of Fc receptor activity and their sizes (as measured by centrifugation layering) changed. This procedure would allow one to tell whether the heterogeneity among these cells is "fixed" or not.

So far we have been discussing the cell cycle's possible control of surface receptors. It is also clear that the reverse relationship occurs: displayed receptors may help to control the cell cycle. ACTH and TSH can act through binding onto surface receptors to cause or permit proliferation of cells in the adrenal[73] and the thyroid[74]. Proliferation and differentiation of B lymphocytes into immunoglobulin-producing plasma cells depends on antigen binding to a surface-bound antibody as the first step. D.B. Thomas[23] looked at cyclic expression of blood group determinants in murine cells and their relation to growth control and found that a cell's ability to express (mouse) blood group determinants B and H on its surface was an "index of its commitment to mitosis." He went on to say -- without offering evidence, we must note -- that "it is reasonable to expect that differences in surface properties of rapidly dividing cells and other cells would be most apparent in G1 and would be minimal in limiting conditions of growth."[24] Neoplastically transformed cells change considerably in their surface membranes including their

membrane receptors[10,26].

The most complete study would be one where the relationship between cell cycle, receptor display, and cell function is fully worked out. One report that comes close to doing this is the work of Revoltella et al.[28] on binding sites for Nerve Growth Factor in synchronized murine neuroblastoma cells. The authors describe Nerve Growth Factor as "a protein which controls the growth of sympathetic cells during development and throughout adult life"; they found that the membrane receptor in these cells became unmasked specifically during late G1 and early S. Although this does not establish a relationship between the display of receptors, Nerve Growth Factor binding, and cell growth, it suggests a method of cell growth control; and the authors point out that their other experiments have shown sympathetic nerve cells to bind the Nerve Growth Factor onto their membrane surfaces to the same extent as neuroblastoma cells.

A provocative study was done in 1971 by Lerner and Hodge[30], who looked at the transition of lymphocytes from the resting to the proliferative stage. It is worth quoting their comments about the conceptual setting of the research: "The reversible transition from a resting to a proliferating cell occurs in such diverse biologic

phenomena as bacterial spore formation, fertilization of the egg, contact inhibition of cultured mammalian cells, and induction and maintenance of the immune response. In lymphocytes the events resulting in cellular proliferation can be categorized conveniently into two fundamental steps: one, the initial 'induction' by immunogen, and two, the subsequent entry of G_o cells into the cell cycle." The investigators found, by using centrifugation to separate cells, that the transition from G_o to G1 was associated with an increase in the number and sedimentation profiles of polyribosomes, an increase in protein synthesis generally and a five-fold increase in the rate of synthesis of immunoglobulin specifically (followed by a decrease in the rate of synthesis of immunoglobulin during S, G2 and M). The increase in immunoglobulin synthesis between G_o and G1 is a true cell cycle-associated phenomenon, and not due to an increased number of immunoglobulin-producing cells having divided. The authors go on to say that it is reasonable to think of a surface receptor for antigen being present during a limited time in the cell cycle (G_o), and of the antigen-receptor interaction inducing the cell to pass through a phase in the cell cycle in which the synthesis of immunoglobulin is obligatory. These ideas provide a plausible picture of the inductive

regulatory system in immunoglobulin-producing lymphocytes.

One system which has been worked out fairly completely, for the interaction of receptor display, cell cycle stage, and cell function, is the activation of the melanizing hormone tyrosinase in cultured mouse melanoma cells. The steps in this activation are (1) binding of melanocyte stimulating hormone (MSH) to a receptor on the cell surface, causing (2) activation of adenylate cyclase, which raises the level of cAMP and cuases (3) an increase in the level of tyrosinase, resulting in an increase in melanin content. Varga et al.[35] determined that the events of MSH-induced melanization distal to the production of cAMP could occur throughout the cell cycle; however, MSH-induced melanization is associated predominantly with G2. In order to understand this G2 preference, Varga and Fritsch examined not only synchronous but also asynchronous melanoma cell cultures for the binding of MSH[33], and found that it was the receptor for MSH that accounted for the G2 association: the receptor itself was present on the membrane throughout the cell cycle, as could be determined readily by unmasking it with neuraminidase; but it was physiologically unmasked and available for MSH binding only during G2.

Further investigation elucidated other significances of the cell cycle for the regulation of melanization. Both

MSH and B_2cAMP, it turned out, could have either stimulatory or inhibitory effects on the growth of melanoma cells[36], the effect possibly depending, for example, on the concentration of cAMP[37]. Varga[34], in an experiment on melanization under conditions of growth in the presence of either of these two compounds, found that MSH not only induced G2 melanization, but also increased the proportion of G2 cells in the population -- an effect that he attributed to the blocking of cells in the G2 phase of growth (as opposed to stimulation of G1 cells); whereas B_2cAMP not only induced melanization predominantly in G1, but also inhibited cells from leaving G1. Thus the two compounds have the dual effects of inducing melanization and of inhibiting cell cycle progress.

These findings suggested the following model for control of melanization, which is the melanocyte's primary function[34]: "(1) cAMP or other agents that increase cAMP levels in G1 induce G1 melanogenesis; (2) MSH or other agents that activate adenylate cyclase in G2, induce G2 melanogenesis." This arm of the control system has to do with MSH receptor display, which is a G2 event. The second arm has to do with the regulation of the cell population by growth controls: "(3) agents which inhibit cells in G1 without the activation of adenylate cyclase may cause the MSH-receptor positive population to become depleted, resulting in a decreased

responsiveness to MSH at the population level. In turn, factors which release cells from a G1 block can have an opposite effect. (4) Growth controls, regulating the duration of G2, may again result in an accumulation or depletion of receptor-positive cells." Thus the cell cycle controls receptor expression, which permits binding, which affects both cell function and (presumably also through the receptor binding mechanism) the cell cycle itself. We can see here the true interaction of cell cycle, receptor and hormone in the regulation of cell function.

The Macrophage and the Fc Receptor

In this project, we wished to look at whether there is a cell cycle association with the display of the Fc receptor on the surface membrane of macrophages.

Macrophages have a central role in immune responses, including both resistance to infection and probably defense against tumors. They act both directly, by ingesting foreign agents, and indirectly, by permitting or potentiating the responses of both B and T lymphocytes. Macrophages are chemotactically attracted to sites of inflammation; through the actions of Migration Inhibitory Factor and Macrophage Activating Factors, they become concentrated at these sites and secrete hydrolytic enzymes and phagocytize and digest debris as well as foreign antigens[17]. In addition, macrophages are important in the induction of cell mediated

immune reactions such as delayed hypersensitivity and the priming of helper T cells which are involved in helping antibody production[17]; for assistance in the process of antigen-induced T lymphocyte proliferation and mediator production[80]; and for assistance in the activation of B cells[81,82].

According to Oliver and Berlin[40], several kinds of receptors are found on the surface membranes of macrophages. These include C3 receptors, which bind antigen-antibody-complement complexes; nonspecific receptors for particles (protein aggregates, latex beads); receptors for plant lectins (such as agglutinins); receptors for Migration Inhibitory Factor; and Fc receptors. Fc receptors are stable in long-term culture and are found on all mononuclear phagocytes[39,40]. They bind immunoglobulins either free or in the form of antigen-antibody complexes, through the C-terminal constant region of the IgG molecule. The presence of these receptors is usually demonstrated by exposing macrophages either to labels of ^{125}I-iodinated immunoglobulins, or to antibody-coated particles (red blood cells) which attach in characteristic rosette forms and are subsequently ingested. They appear to play a key role in the immunobiology of these cells. (They are found also on polymorphonuclear leukocytes, B cells, some T cells, mast cells, and herpes virus-infected cells[48].) By binding to the Fc portions of

antibody molecules which project from opsonized bacteria or viruses, the Fc receptors facilitate the process of particle ingestion or phagocytosis; and by binding antibody-coated antigen, they enable the macrophage to present antigen to B and T cells, thereby initiating their responses.

The study of macrophages demands special materials, because macrophages cannot yet be separated in a completely uncontaminated population from other cell types. Thus, for work on a pure macrophage population it is necessary to use one of the cultures of tumor lines that have macrophage-like characteristics. We used P388D1 cells, which are commonly investigated for macrophage functions and Fc receptor properties and binding. This line was isolated at the National Cancer Institute, was passaged for many years in tissue culture, and was determined by Koren et al.(6) to possess many macrophage-like characteristics, including (1) Fc and C3 surface receptors (2) intracytoplasmic nonspecific esterase (3) the ability to phagocytize polystyrene particles (4) firm adherence to glass and plastic surfaces, and (5) a lack of surface immunoglobulin. In addition to these characteristics the cells were found to have high effector cell activity for the antibody-dependent-cell-mediated cytolytic reaction. Generation time is about

20 hours when the cells are grown in Eagle's Minimum Essential Medium with 10% heat-inactivated Fetal Calf Serum. Finally, P388D1 cells' ease of growth and manipulation make them excellent for experimental work.

There have been a number of recent discoveries about the importance of Fc receptors to macrophage immune function. Hurwitz et al.[57], investigating the antibody-dependent cell-mediated cytolytic reaction, found that among mouse normal spleen cells, macrophage-like cells, and lymphocyte-like tumor cells, those which bore Fc receptors on their surfaces were effective mediators of the reaction, whereas those not bearing Fc receptors were inactive. Pierres et al.[44] determined that in responder mice, macrophages which are able to present antigen in an immunogenic form play a central role in regulating the balance of activated helper and suppressor T cells. Ptak et al.[43] discovered that mouse peritoneal exudate macrophages could transmit T cell-derived suppressor signals to other T cells, and that the receptor on the macrophage for this antigen-specific signal factor had Fc receptor-like characteristics; the authors speculated that the presentation of such signals to T cells might be as important a role for the macrophage as is the presenting of antigen. Soulillou et al.[45] found that Fc receptors may be involved in graft rejection through a role in aiding

T and B cell collaboration. And Kerbel, in a review[46], discussed a disadvantageous aspect of Fc receptors: the induction of the receptors after herpes virus infection on the surfaces of every type of cell thus far tested in six different species, and the possible role of the receptor in potentiating the viral infection by protecting against destruction of the cells. We would seem to be only at the beginning of learning the functions of the Fc receptor.

The Fc receptor appears to be a lipoprotein complex[50] that is distinct from the receptor for C3[51]. Isolation and biochemical characterization has only recently been begun, and results vary both because of differences in methods of isolation and because of the likelihood that there is more than one type of Fc receptor (see below). Loube et al.[55] used for isolation monomeric mouse IgG2a (which we used for our experiments) and monomeric human IgG1 on Sepharose columns with detergent-solubilized P388D1 lysates; they obtained what appeared to be single polypeptide chains of molecular weights 57,000 (major band), 28,000 and 24,000 (minor bands) which represented either all or some portion of the Fc receptor.

There appear to be at least two different kinds of macrophage Fc receptor: one ("monomeric") specific for monomeric and aggregated IgG2a and sensitive to trypsin, and another that binds immune complexes or aggregates of

IgG1, IgG2a, and IgG2b and resists trypsin digestion[48]. Anderson and Grey[49] have physically separated the two kinds of Fc receptor from detergent lysates of P388D1 by using affinity chromatography and sucrose gradient centrifugation. The authors suggested, but have not demonstrated, that the receptors might possibly serve different immunologic functions. In all but one of our own experiments we used monomeric IgG2a, so presumably we were obtaining binding to only the "monomeric" receptor.

The binding properties of the P388D1 Fc receptor were studied by Unkeless and Eisen[56]. The receptor bound IgG2a much more strongly than IgG2b; whereas it did not bind IgM, IgA, or IgG1 significantly at all. The K_a for one of the IgG2a molecules (different from the one we used) was 1.3×10^8 M^{-1} at 4^oC, and binding was exothermal. It was specific for determinants in the constant region of the molecule. Finally, the receptors were readily eliminated by brief exposure to trypsin, and regenerated (60% in 12 hours) during subsequent cultivation in serum-free medium; regeneration was inhibited totally by treatment with cycloheximide or Actinomycin D, demonstrating that the receptors are produced by the cells that display them. Segal and Hurwitz[43] also investigated the receptor's binding properties, and using UPC-10 at 30^oC (the same immunoglobulin

as ours, but at a lower temperature) obtained a K_a of between 2.3 and 8.1 x 10^6 M^{-1}. Both Unkeless and Segal found the number of binding sites per cell to be in the neighborhood of 1 x 10^5.

Monomeric vs. Aggregate Binding

These findings about the Fc receptor's binding properties lead straight into an apparent paradox. One conclusion drawn by Unkeless and Eisen[56] is that, given the affinity of the mouse macrophage Fc receptor for IgG2a and the physiologic concentration of this molecule in the plasma, probably the membrane Fc receptors are saturated *in vivo*. This raises the question of how the macrophage can preferentially bind ("distinguish" so to speak) antigen-bound antibodies (those in immune complexes or on opsonized particles) from free monomeric antibodies, as would seem necessary for the macrophage to function immunologically. Two theories have been tested: one, that there is a qualitative difference between the binding affinities of free monomers *vs.* antigen-antibody complexes, such as might be caused by a steric change in the Fc region upon antigen binding; and two, that there is a difference between the affinity for monomeric *vs.* oligomeric immunoglobulins (with the latter being more likely to be involved in immune function).

Studies have strongly supported the second hypothesis. Phillips-Quagliata et al.[38] found that the amount of binding of antigen-antibody complexes to macrophages was greatest, first, when there were at least two valences present on the hapten, and second, when there was an antigen to antibody ratio value between equivalence and a slight antibody excess, which condition favors lattice formation; they also found that almost complete inhibition of binding of the antigen-antibody complex was obtained in the presence of sufficient immunoglobulin monomer. This experiment suggests both that monomeric immunoglobulin binds less strongly than multimeric, and that the binding of uncomplexed and unaggregated (monomeric) immunoglobulin is not dependent on an allosteric change in either the Fc region or the Fc receptor; however, it leaves open the question whether possible allosteric changes may play any role at all.

Several subsequent studies have explored the nature of the enhanced binding by oligomers. Knutson et al.[29] showed with heat-aggregated IgG's that the equilibrium constants of binding increased directly with the increase in size of the aggregates; this binding was inhibitable up to 50% with monomeric immunoglobulin at physiologic concentrations. Segal and Hurwitz[42] examined the binding to macrophages

of IgG monomers and covalently cross-linked oligomers. Their equilibrium studies showed that trimer bound more strongly than dimer, which bound more strongly than monomer. Kinetic studies showed that for both dimer and trimer there was a fast and a slow reaction of both binding and dissociation. The authors determined the free energies of binding for the oligomers, and interpreted these to show that among the immunoglobulin monomeric subunits within oligomers, the first subunit bound much more strongly than the second, and the second more strongly than the third. This is based on their assumption that the difference in free energy of binding between trimeric, dimeric and monomeric immunoglobulin molecules is equivalent to the difference between subunits of the trimer. If this interpretation of the data is correct, it supports their idea that the binding of oligomers occurs not all at once but in several stages, with binding first to one subunit and then sequentially to the other subunits. The total equilibrium constant for each molecule would then be the product of constants for each step. This hypothesis can explain both the greater affinity of binding for oligomers, and the biphasic association and dissociation curves. Furthermore, it explains this difference in affinities without invoking a steric change in the Fc region upon binding, which would contradict

the finding that monomeric immunoglobulin can inhibit the binding of antigen-antibody complexes. With respect to this last point, however, one must note that Segal and Hurwitz worked with oligomeric immunoglobulin molecules that were not complexed to antigens.

In their experiment, Segal and Hurwitz found concentration-related partial inhibition of oligomer binding by monomer, at much less than physiologic concentration of monomer. As they point out, the discovery that oligomers bind more tightly, and the multistage binding mechanism which they propose to explain this phenomenon, still do not solve the problem of how it is determined that macrophages will preferentially bind oligomeric antigen-antibody complexes: the competition from monomers would appear to be simply too great. The authors do suggest a mechanism whereby such "distinctions" might be achieved by B lymphocytes, where the displayed surface immunoglobulin molecules are specific for an antigen. The problem in macrophages, however, remains to be solved. One might look in several directions for a solution. A first step might be to try to repeat the finding of Phillips-Quagliata et al.[38] that uncomplexed monomeric immunoglobulin can completely inhibit the binding of antigen-antibody complexes, and to repeat it using antigen-bound monomer and not only uncomplexed monomer as the potential inhibitor. The purpose of this

would be to try to prove that the binding of the antigen to the antibody molecule does not affect its affinity for the receptor or the receptor's affinity for it -- a point which is not yet sufficiently established. A second consideration is whether the "discrimination" can be related to the difference in types of Fc receptor. A possibility to be included here is that one might question whether the different types of receptors may not be different forms of the same basic receptor, altered by a previous binding event. Another possible mechanism that could explain the difference in affinities is a redistribution of the surface Fc receptors upon binding of an oligomer, so that the dissociation (or even the completion of the association) would favor the oligomeric subunits because of proximity of the receptors. It has already been shown that some capping of Fc receptors occurs with binding[27]. Although this demonstrates redistribution only in a gross way, the finding encourages pursuit of this line of investigation. Whether the cell cycle is involved at all in control of this discriminatory aspect of binding, as well as in the number of receptors displayed, is a question that our experiments did not ask; however our photographs showing nonrandom binding are relevant (see below).

In our experiments we were concerned with both the binding properties of Fc receptors and the possible control

mechanisms underlying their display on macrophages. To review, we have seen in the literature that the display appears to be increased in Macrophage Growth Factor-stimulated macrophages[32] and in peritoneal exudate macrophages[21] over unstimulated macrophages. Rhodes has found two other associations: that there is an increased number of Fc receptors on macrophages in a variety of malignancies[26], and that cAMP and insulin block the increase in Fc receptor expression which normally occurs with cell growth[59]. This last finding can be considered together with those of Varga[34] and Froelich[4] who found in other systems that an increased level of cAMP inhibited progress through the cell cycle. In that context it suggests the possibility that the number of Fc receptors per cell in mouse macrophages bears a relationship to the cell cycle.

One final finding to note is that binding of hormone, mitogens, and immunoglobulins to the cell surface receptors has been found, in at least two systems, to be discontinuous at least some of the time. Garrido[19], we recall, found greater discontinuity in binding of Concanavalin A and Wheat Germ Agglutinin to Chinese Hamster Ovary cells in mitosis than in interphase; and Varga et al.[8] discovered that MSH receptors on the surface of mouse melanoma cells were displayed in clusters, possibly associated with the Golgi apparatus or some other cell organelle. Finally, Romans

et al.[27], who examined the binding of immunoglobulin-coated erythrocytes to human blood monocytes and peritoneal macrophages, found there to be capping; it was time-, temperature- and metabolism-dependent, and therefore represents an active process of redistribution of the Fc receptors with binding. Whether the capping phenomenon also occurs with monomeric ligands was not examined at the time.

DNA CONTENT vs. ANTIBODY BINDING

Summary

In a group of experiments, we established conditions for our subsequent work of investigating the amount of binding of IgG to the surface receptors of mouse macrophages at different times in the cell cycle. Using an iodinated IgG2a as a marker on P388D1 cells in culture, we determined the following: (1) that binding is essentially complete by 15 minutes; (2) that at levels of less than complete confluence, cell density does not affect binding; (3) that our iodination procedure does not destroy the IgG molecule's capacity to bind; and (4) that our results for binding kinetics (apparently first-order, with a K_D of 5.2×10^6 M^{-1}) and receptor density on the cell surface (3×10^5 sites per cell) agree well with values reported in the literature. We then used the double Thymidine block technique and a technique of autoradiography of Feulgen-stained nuclei to examine the relationship between the cell cycle and the amount of binding. The results suggest that binding increases as cells progress through the cycle.

Photographs of autoradiographed cells show nonrandom binding of the antibody to the cell surface.

Introduction

In the previous chapter's discussion, we saw that the control of cell functions may involve an interaction between the cell cycle and the display of surface membrane receptors. We wished to look at the relationship between cell cycle stages and the display of Fc receptors on the surface of mouse macrophages. In our first experiments we established the reliability of our materials and methods by showing agreement with values reported in the literature. We then used the double Thymidine block technique, which is a well-established though not perfect method for synchronizing cells in culture[62,63], to take an initial look at the cell cycle vs. binding relationship.

An improved method for determining both DNA content and antibody binding became available during the course of this work. A more refined and rapidly-operating microfluorimeter, which reads the fluorescence of Feulgen-stained nuclei, enabled us to make many readings in a short time; and a bubble method of applying an ultrathin layer of nuclear track emulsion onto the surfaces of cells labelled with ^{125}I-iodinated immunoglobulin[8] made it possible to record, simultaneously with DNA content, the number of cell-associated grains that reflected binding. The advantages of these methods were, one, they did not

interfere with cell metabolism; two, they did not depend on synchronization; and three, they permitted direct visualization of the binding pattern on cells. In preparation for this work, we looked at different methods of Feulgen staining, to see which would give the clearest separation of G1 and G2 peaks in a nonsynchronized population. Fujita reported a variation of the Feulgen staining method[9] which we tested against both the standard method and a variation of his variation.

Fujita's method gives a clearer resolution of the G1 and G2 peaks in a population of cells, we verified, than does the standard method. We then employed this Fujita variation along with the technique of reading exposed emulsion grains (associated with nuclei) to look at the association between Fc receptor display and progression through the cell cycle.

Materials and Methods

Culture and Preparation of Cells:

P388D1 mouse macrophage cells were obtained from Dr. P. Ralph and Dr. M. Horowitz and were cultured in 75cm^2 Falcon tissue culture flasks in a 5% CO_2-95% air atmosphere; Dulbecco's Minimum Essential Medium Eagle (DMEM) was supplemented with 10% Fetal Calf Serum and 50 micrograms/ml of Gentamycin.

For experiments on binding conditions and kinetics,

cells were taken up with a rubber policeman and transferred to 25cm^2 flasks; they were left for at least 6 hours before experimentation, to allow them to attach to and spread out on the flask surface.

Cultures for determination of DNA content were grown by seeding approximately 5000 cells in 2 ml of medium onto cleaned and heat-sterilized glass coverslips (Corning 22 x 22mm) placed in Limbro tissue culture multi-well plates.

Preparation of Label:

This procedure was based on Unkeless'[56] version of Sonoda[65].

Purified UPC-10 mouse myeloma protein, obtained from Bionetics Co., was further purified by chromatography on a Sephadex G-25 column in phosphate buffered saline. The eluted fractions were read for optical absorbance at 280 nm and the three fractions with the highest absorbance were pooled.

To iodinate, approximately 0.1 mg of the UPC-10 (or of Fc trimer, which had been obtained from Dr. William Barnes[61] and purified as above) was reacted with one mCi of ^{125}I (obtained from New England Nuclear Co.) in the presence of 10 microliters of Chloramine T (0.4 mg/ml) for one minute, and the reaction was then stopped with 50 microliters of 0.04% tyrosine and 10 microliters of 1 M KI. The labelled immunoglobulin was chromatographed

in phosphate buffered saline on a Sephadex G-25 column and it appeared in the void volume. (Among different iodinations there was some variation in the amounts of protein and iodine and in the duration of reaction.)

The solutions to be used for incubation with cells were prepared by diluting the stock of labelled immunoglobulin with different amounts of unlabelled immunoglobulin and/or phosphate buffered saline with 2 mg/ml of bovine serum albumin, fraction V (PBSA).

Labelling and Assays:

Flasks were washed twice with 10 ml of Hanks Balanced Salt Solution at 37°C. They were then incubated for two hours at 37°C in serum-free MEM containing 0.05% hydrolyzed lactalbumin. Then the cells were put on ice for at least 5 minutes, the solution was aspirated and it was replaced by a 1:1 solution of 0°C PBSA:L-15 culture medium containing the desired concentration of ^{125}I-labelled immunoglobulin. The cells were incubated with this label for a specified period of time. At the end, the flasks were washed three times with 35 ml of PBSA at 0°C and the cells were taken up with a rubber policeman.

Radioactivity of each sample was counted in a Coulter Counter (model ZB1). The suspensions were then centrifuged at 800 rpm for 10 minutes. Cells were resuspended in 10 ml of "Isosol" and counted in a S/P AW-1450 Gamma

Counter.

For the autoradiography experiments, the procedure was varied: after the cells had had at least six hours to attach to the coverslips, the wells were rinsed with 2 ml/well of Hanks Balanced Salt Solution at 37°C and incubated two to three-and-a-half hours with serum-free MEM containing 0.05% hydrolyzed lactalbumin. They were then rinsed with 2 ml/well of 1:1 MEM:PBSA at 0°C and the Limbro plates containing the coverslips were placed on ice. We then placed 0.1 ml of prepared label on each coverslip, taking care to avoid spilling the radioactive solution onto the well itself. The Limbro plates were incubated on a flat surface at 0°C for one hour.

The labelling solution was removed from the coverslips in several steps, in order to reduce residual radioactivity to a minimum. First the fluid was aspirated from a corner of each coverslip while the plates remained flat; then the plates were tilted and again fluid was aspirated, from a corner or side; then each well was rinsed twice (15 seconds each time) with 2 ml of phosphate buffered saline at 0°C; and finally the slides were placed in a holder to be washed together in five one-liter volumes of phosphate buffered saline at 0°C for three minutes each wash, with careful removal of excess fluid from the slide holder upon each transfer. Cells were fixed for five minutes with 2% para-

formaldehyde and air-dried.

Autoradiography and Microphotography:

A Kodak OC filter safelight was used in the darkroom. From Ilford L4 nuclear track emulsion at 45°C approximately 10 microliters were withdrawn with a micropipette fitted with a mouthpiece. For each coverslip a bubble about one-and-one-half inches in diameter was formed on a flat, clean cellulose acetate surface, and the coverslip was lowered face down onto this bubble to give a very thin coating of emulsion about one inch in diameter. Coated cells were kept in a light-tight box for three to six days. The gold-EAS method[75] was used to develop the slides, in complete darkness so as to reduce extraneous exposure.

For photographing cells, we stained one coverslip for one hour with Giemsa in the manner described by Humason[76], using Giemsa stock, 100% methanol, distilled water, 1/10 M Citric acid and 1/5 M disodium phosphate in a ratio of 2.5:3:100:11:6. All photographs in color were taken with Kodak Ektachrome 160 Tungsten color slide film under either fluorescence or white light, as indicated. Black-and-white photographs of Feulgen-stained cells were taken with Kodak Tri-X.

Cell Synchronization:

Flasks and coverslips were treated by double Thymidine

block simultaneously as follows: (from Mitchison[7])

(a) cells cultured as above

(b) culture for 16 hours with medium containing Thymidine 2×10^{-3}M

(c) medium replaced with normal medium (i.e., no excess Thymidine) and culture for 10 hours

(d) culture for 14 hours with medium containing Thymidine

(e) Thymidine block released by replacement with normal medium

Cell Staining and Measurement of DNA Content:

For the initial experiments measuring DNA content, cells on coverslips were fixed in Carnoy's fixative and stained by the Feulgen procedure. The DNA content of the nuclei was then measured in a Zeiss type 05 scanning microspectrophotometer at 560 nm using a Wang 700 calculator operating Wang program 3967. Each area of scanning was selected at random and all nuclei visible in the field were read.

For the experiments using cytofluorimetry, an initial test of different staining methods was done: after fixation in Carnoy's solution for 5 minutes at room temperature, and then rehydration through 100%, 95%, and 70% ethanol and distilled water (two minutes each), three different staining procedures were followed:

(a) Standard method: 5N HCl for five minutes; then rinse with 0.01N HCl for one minute; then Schiff reagent one hour at room temperature.

(b) Fujita's method: 1 N HCl at 60°C for five minutes; then rinse in 0.01 N HCl for one minute; then Schiff reagent, diluted to 0.05% with $Na_2S_2O_5$ buffer and adjusted to pH 2.7, for ten minutes at 7°C.

(c) Variation of Fujita's method: 1 N HCl at 60°C for five minutes; then rinse in 0.01 N HCl for one minute; then undiluted Schiff reagent at 7°C for ten minutes.

All slides were rinsed with bisulfite solution and with water and dehydrated in the standard manner.

In subsequent experiments the "B" method was used for staining.

Cytofluorimetry:

After dehydration the samples were mounted on slides with a DIN 58 884 (Leitz) low-background immersion oil. A Leitz MPV 2 microfluorimeter was used, equipped with a 200 W Xenon burner and a "Pleomopak 2.2" vertical illuminator with BG 36 and KP 560 and K 530 exciting filters, TK 580 dichroic beam splitting mirror and K580 suppression filter.

In the first experiment, a group of 220 randomly

selected cells and of 100 labelled cells were read for absorbance, and the results plotted with absorbance as the independent variable. In succeeding experiments, cells were read regardless of whether they were associated with granules, and we attempted to avoid reading repeatedly from the same area of any slide. Because of the great variation in grain distribution between slides, and among different areas on any single slide, only those areas were read where there were clearly grains associated with some cells, and where the number of background grains (in a cell-free area) did not exceed approximately two in an area approximately 20 times that of an average nucleus. (It was not always possible to find such a low background.) For each cell we recorded the fluorescence intensity (to the nearest tenth) and the number of grains on the nucleus or within one nuclear diameter's distance from it.

Results

In our first experiments we determined that binding of the immunoglobulin was essentially complete by 30 minutes, which agrees with the literature[56]; and that there was no discernable effect of cell density upon binding, within the moderate range of densities that we were using.

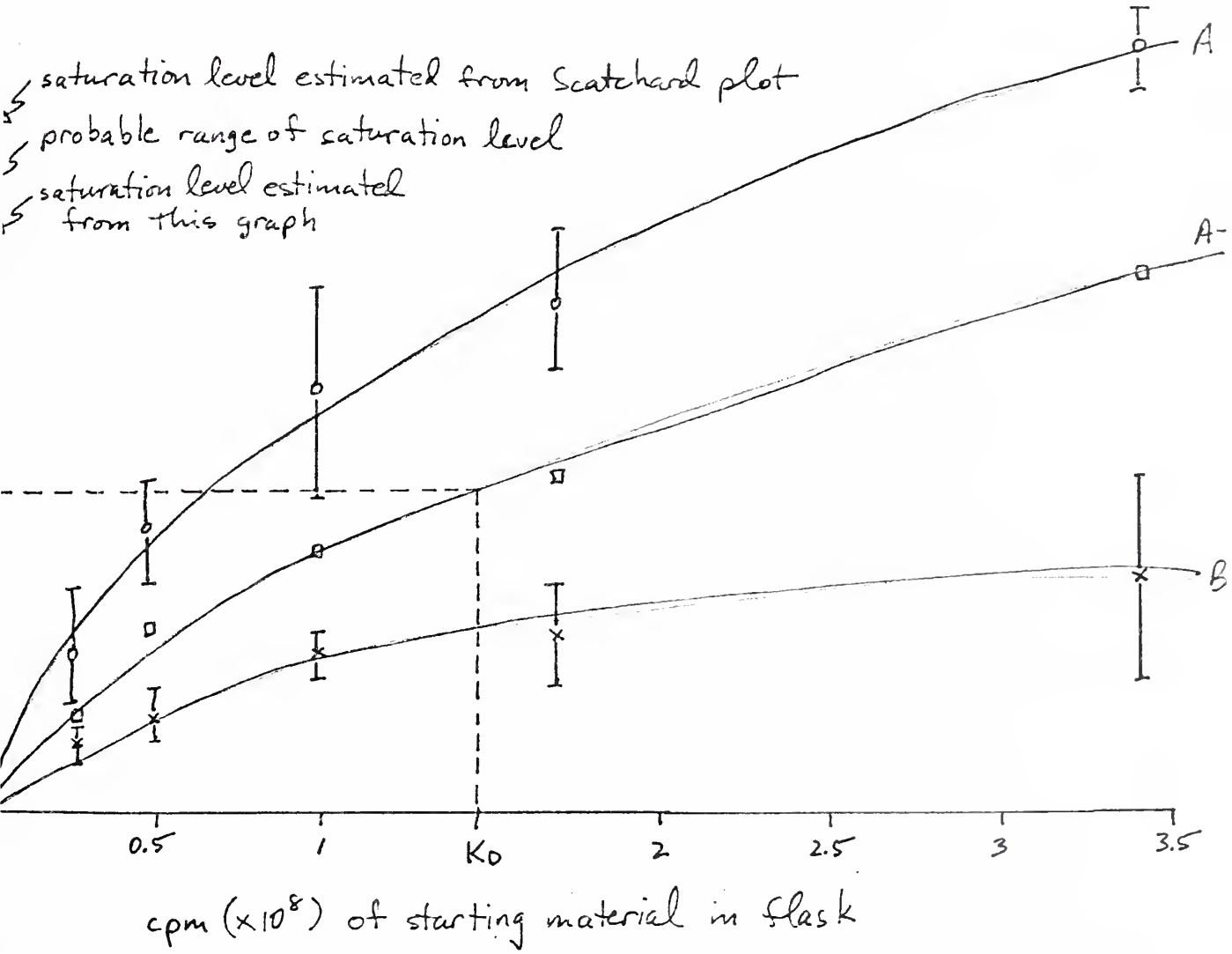
Binding Kinetics:

In order to investigate the binding kinetics of the labelled immunoglobulin, and receptor density per cell, we incubated cells with labelled immunoglobulin at various concentrations under conditions of 10-fold (A) and 100-fold (B) molar excess of unlabelled immunoglobulin. Figure 1 shows the binding curves for the "A" and "B" conditions, and an "A-B" curve which presumably represents receptor-associated, as opposed to nonspecific (because not able to be specifically competed) binding. Clearly the binding of labelled antibody is suppressed by the higher concentration of unlabelled antibody in the "B" solutions. This is evidence that iodination has not destroyed the immunoglobulin molecule's binding capacity.

The results allow calculation of the number of binding sites per cell and the K_D as follows: from Figure 1, where the "A-B" curve appears to be levelling off we can estimate saturation level of immunoglobulin (r) to be approximately 17 to 20 cpm x 10^4. A Scatchard plot of the data (Figure 2) extrapolated to r/c=0 (c theoretically being infinite, in this case) yields r = 20 x 10^4 cpm. Finally, a plot of 1/r vs. 1/(free) (Figure 3) extrapolated to (free)=∞ shows 1/r = 0.5 x 10^{-5}, which gives r = 2 x 10^5 cpm. (Note that r is an absolute amount of radioactivity, not a

Figure 1

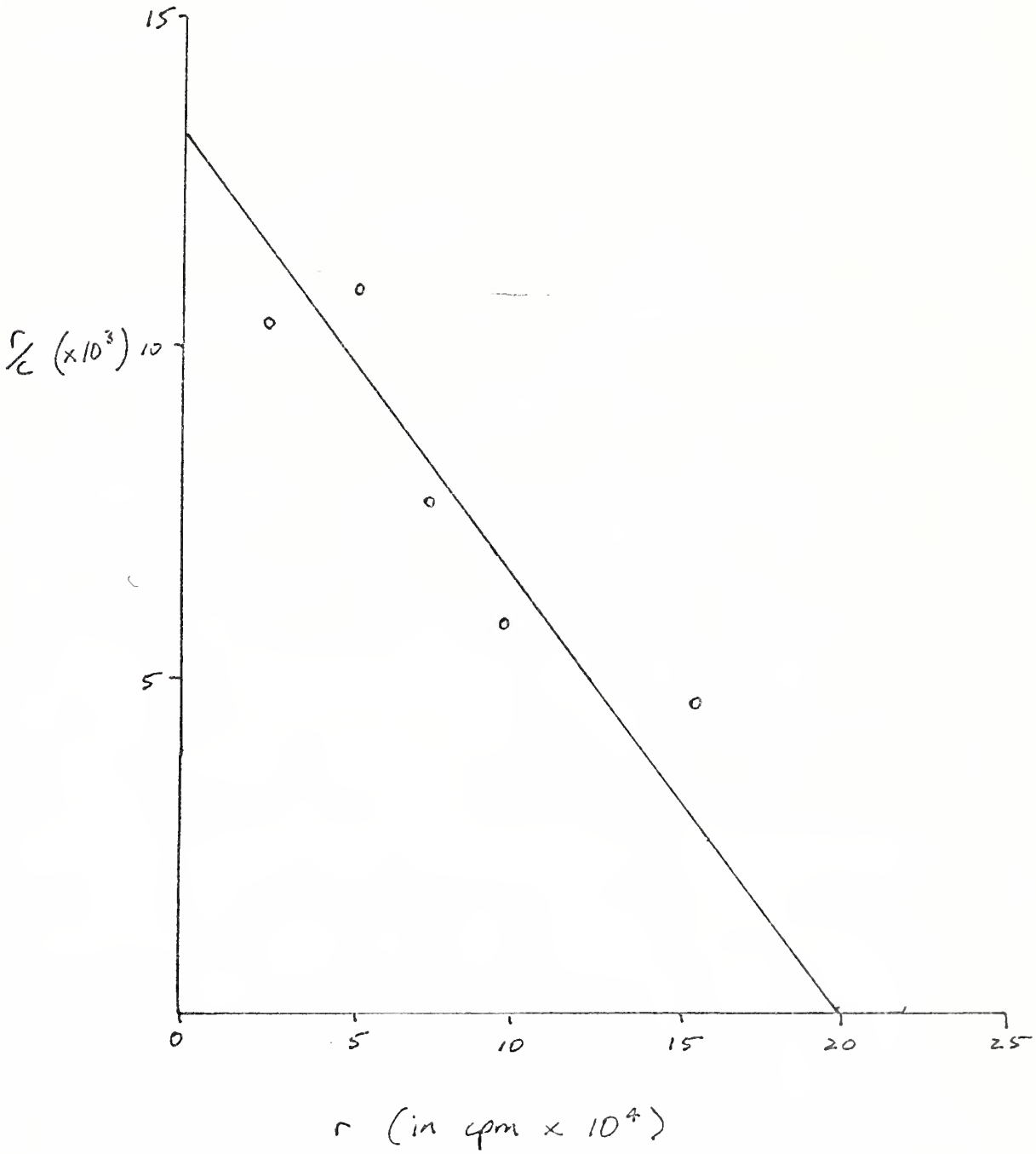
Binding vs. Concentration Under Competitive Conditions



These plots show binding of labelled immunoglobulin at various concentrations under conditions of (A) 10-fold and (B) 100-fold molar excess of unlabelled immunoglobulin.

Figure 2

Scatchard Plot of Data From Figure 1

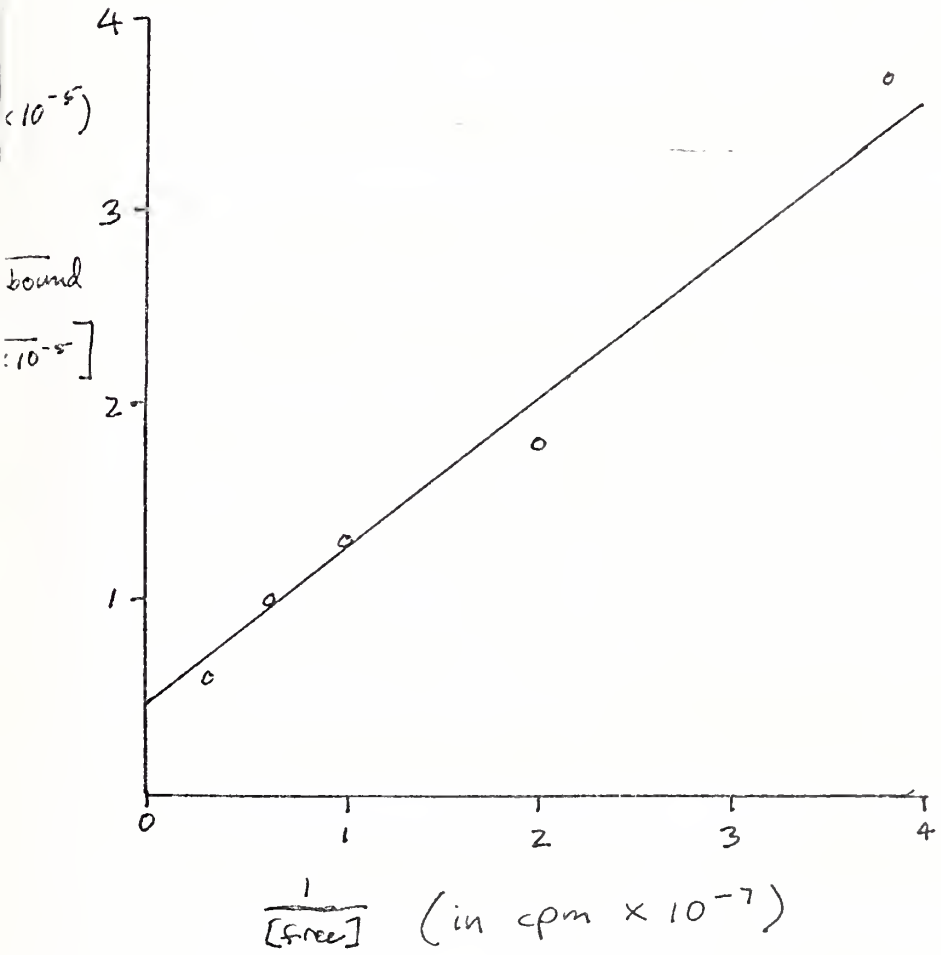


r = A-B (radioactivity of flask fluid)

c = radioactivity of starting material

Figure 3

Reciprocal Plot of Data From Figure 1



concentration.) The initial concentration of labelled immunoglobulin was approximately 4×10^{-8}M = 4×10^{-11} moles/ml. Initial radioactivity was 3.3×10^{8} cpm/ml. Assuming 5×10^{5} cells per flask, we can calculate the number of binding sites per cell to be:

$$\frac{\text{initial molar conc. of Ig} \times 6 \times 10^{23}}{\text{initial radioactivity}} \times \frac{\text{saturation radioactivity}}{5 \times 10^{5} \text{ cells}} =$$

$$\frac{4 \times 10^{-11} \text{moles/ml} \times 6 \times 10^{23}}{3.3 \times 10^{8} \text{ cpm/ml}} \times \frac{2 \times 10^{5} \text{cpm}}{5 \times 10^{5} \text{ cells}} =$$

3×10^{5} molecules/ cell (or sites/cell)

The dissociation constant, K_D, can be estimated from Figure 1 as indicated: we use the approximate value for saturation of 18.5×10^{4} (as obtained from this graph and the Scatchard plot); we take a value of 9.2 for half-saturation; and we find the corresponding concentration value of 1.5×10^{8}cpm/ml. Then

$$K_D \quad \frac{\text{initial conc. of Ig in moles/L}}{\text{initial conc. of Ig in cpm/ml}} \times \begin{matrix}\text{(conc. in cpm/ml at} \\ \text{1/2 saturation)}\end{matrix} =$$

$$\frac{4.2 \times 10^{-7}}{3.3 \times 10^{8} \text{ cpm/ml}} \times (1.5 \times 10^{8}\text{cpm/ml}) = 1.9 \times 10^{-7}\text{M}.$$

Then $K_o = 1/K_D = 5.2 \times 10^{6}$ M^{-1}.

Another result of this work is to show that the binding curve is hyperbolic, thereby suggesting (although not proving) that we are observing a first-order reaction such

as we expect of a solution of molecules reversibly binding to a receptor. The evidence for a hyperbolic function is that the reciprocal plot is linear. Assuming that the graph expresses a binding phenomenon, we can derive the equation and its reciprocal as follows:

(R) free receptor concentration
(L) free ligand concentration
(RL) concentration of ligand-receptor complex

$$(R) + (L) \rightleftharpoons (RL)$$

$$K_D = \frac{(RL)}{(R)(L)}$$

$$(R) = 1 - (RL)$$

$$(RL) = (R)(L)K_D = K_D(L)\left[1 - (RL)\right]$$

$$= K_D(L) - K_D(L)(RL)$$

$$\frac{1}{K_D} + (L) = \frac{(L)}{(RL)}$$

$$(RL)\left[\frac{1}{K_D} + (L)\right] = (L)$$

$$(RL) = \frac{(L)}{\frac{1}{K_D} + (L)} = \frac{1}{\frac{1}{K_D(L)} + 1}$$

This is the equation $y = \frac{1}{\frac{1}{x} + 1}$, and it is a hyperbolic function. The reciprocal equation is $\frac{1}{y} = \frac{1}{x} + 1$, and it is linear.

In sum, the immunoglobulin binding activity here appears to show first-order kinetics; and it reproduces two major

characteristics of binding that are in the literature on Fc receptors, namely the K_D and the number of receptor sites per cell. (43,56)

Monomeric vs. Oligomeric Binding:

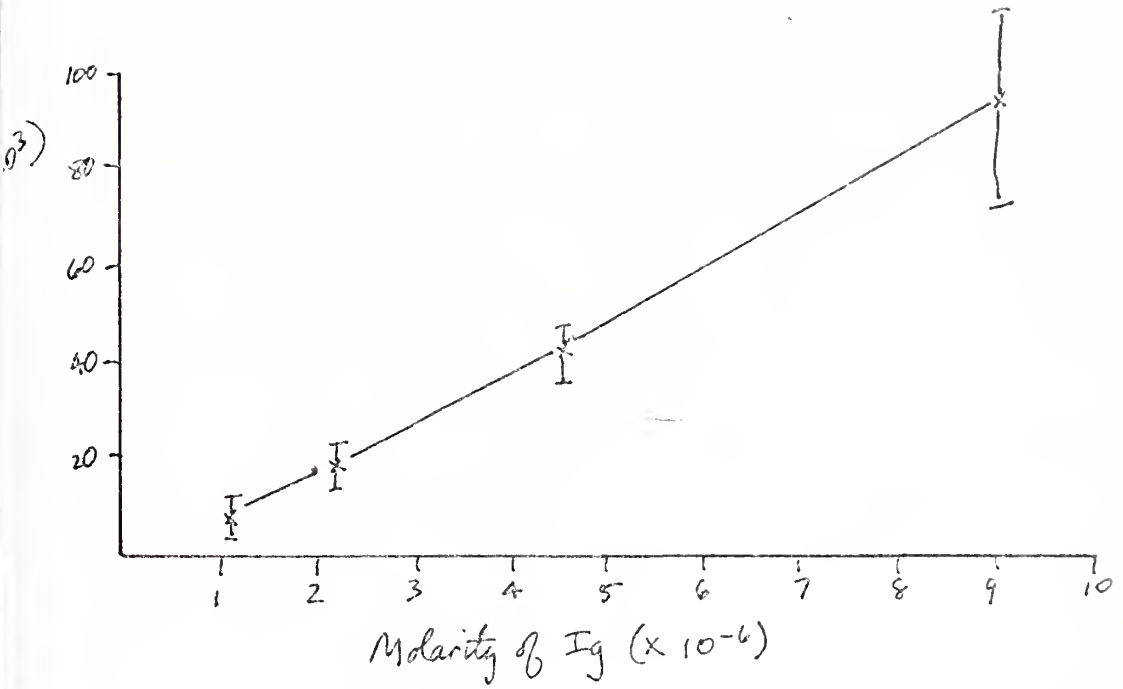
The next experiment was an attempt to duplicate the work of Segal and Hurwitz(43), who showed that covalently cross-linked oligomers of rabbit IgG bound to P388D1 cells with varying affinities: greatest for the trimer, less for the dimer, and least for the monomer and for UPC-10. Figures 4 and 5 show an increase in binding with increasing concentrations of immunoglobulin, but neither curve demonstrates, by flattening out, that saturation has been neared. Thus, we were not able to establish conditions that might have shown a correspondence to the results of Segal and Hurwitz, who demonstrated such a flattening of their curve at a concentration of approximately 1×10^{-8} M for each immunoglobulin. We cannot say anything about the relative affinities of the binding of the two proteins that we used.

Binding vs. Time In Synchronized Cells:

Having established conditions for incubating cells with labelled UPC-10, we proceeded to the first experiments involving the cell cycle as a parameter. The technique most feasible at the time of this work was culture synchronization with double Thymidine block(62,63), which

Figure 4

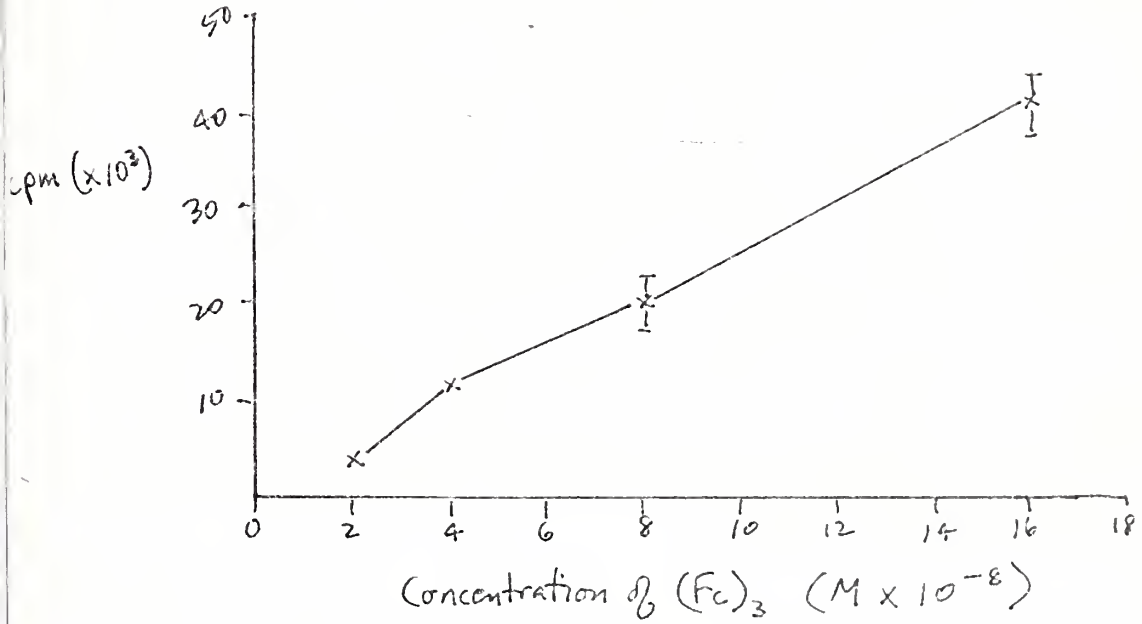
Binding vs. Concentration of UFC-10



Cells were incubated with a solution of labelled and unlabelled immunoglobulin at 0°C for one hour, washed, and then dissolved in 1M NaOH. Radioactivity of aspirated fluid was measured to determine the amount of binding.

Figure 5

Binding vs. Concentration of $(Fc)_3$



Cells were incubated with a solution of labelled and unlabelled $(Fc)_3$ at $0^{\circ}C$ for one hour, washed, and then dissolved in 1M NaOH. Radioactivity of aspirated fluid was measured to determine the amount of binding.

arrests cells at the G1/S interface until release.

As a first step, we used microspectrophotometry to check the members of the cell populations for DNA content, and found the expected distributions of DNA content values for synchronized populations sampled at different times after release from the block. We then used the same synchronizing technique along with the labelling assay for immunoglobulin binding. There appeared to be roughly a 30% increase in binding from the time of release from Thymidine block to four hours later, with a plateau of high binding through approximately ten hours later. During this time the number of G1 cells decreased by about 30% and the number of G2 cells increased about twofold.

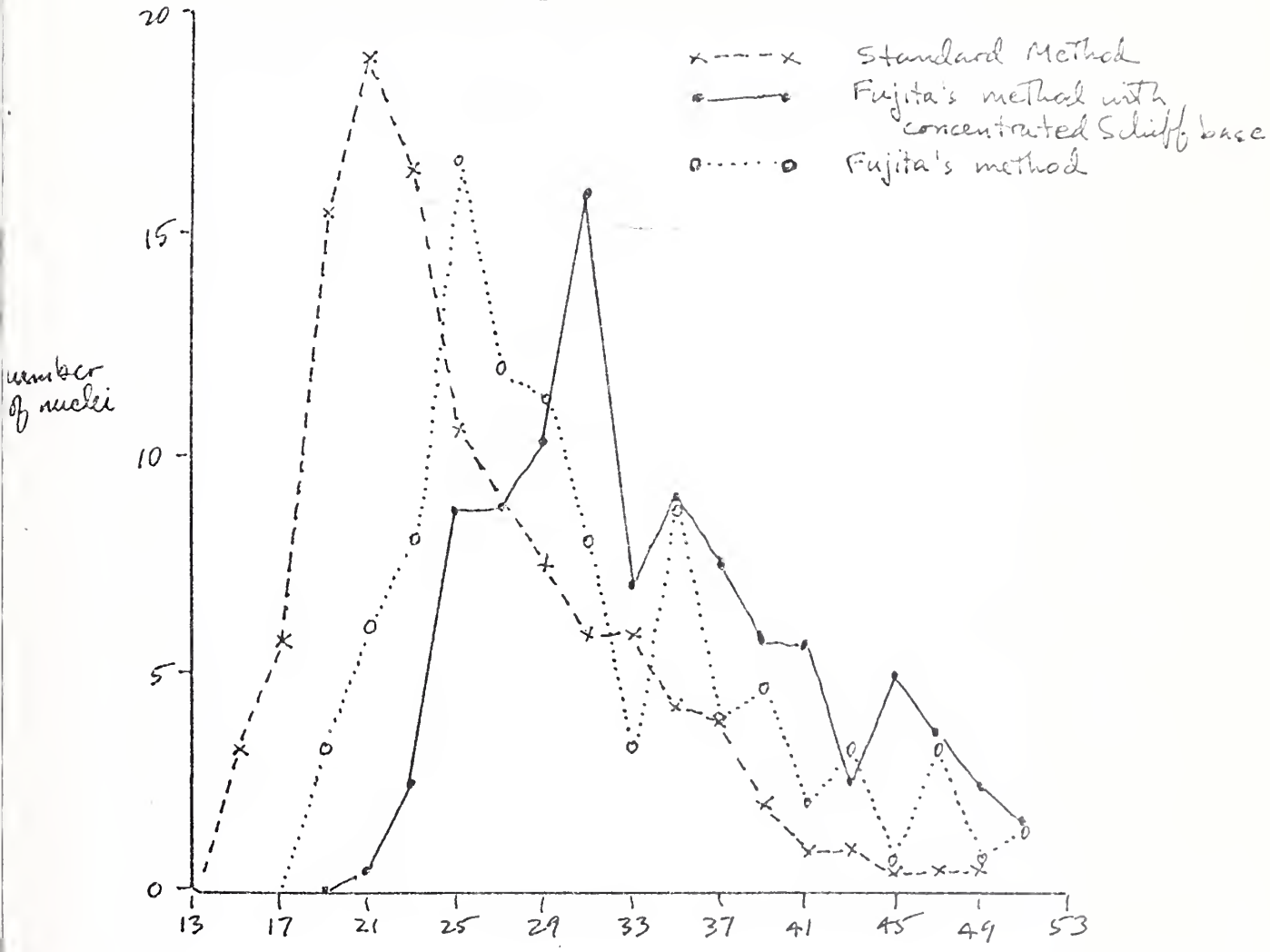
Comparison of Staining Methods:

For the comparison of the standard Feulgen staining procedure and two variations, data were grouped by units of two, normalized, and plotted in Figure 6.

The standard method of staining shows a G1 peak, but does not resolve the expected G2 peak at all. Fujita's method shows more scatter and it also shows a G2 peak. The G2 peak is not distinct, as is to be expected because there is also an S population, but it is to be found somewhere between 35 and 50 -- closer to 50 because of the S cells. Since the G1 peak is at 25, this method

Figure 6

Fluorescence vs. Cell Frequency for Three Different Methods of Cytofluorimetric Staining



Feulgen-stained nuclei on coverslips were read under standardized conditions on a microfluorimeter. x-axis values are in units of fluorescence.

yields roughly the expected 2:1 ratio of DNA contents between G2 and G1 populations. The concentrated variation of Fujita's method does not resolve the G1 and G2 peaks any more clearly.

The Fujita variation was used in subsequent experiments.

Autoradiography:

Visual results of the binding experiments are shown by the following photographs. An example of the very best results of our procedure is the cell in Figure 7, which was Giemsa stained and photographed through a blue filter. The nucleus stands out as a dark cicle on a paler blue background, amidst large dark dots and strands of dust; and the exposed grains of the emulsion show as small dark spots on the circumference of the nucleus.

Figure 7

Another photograph of the same cell with fluorescent light (Figure 8) shows the bright grains and some of the irregularity of nuclear texture. The bright spots in this photograph and the dark/silver grains in Figure 7 are identical.

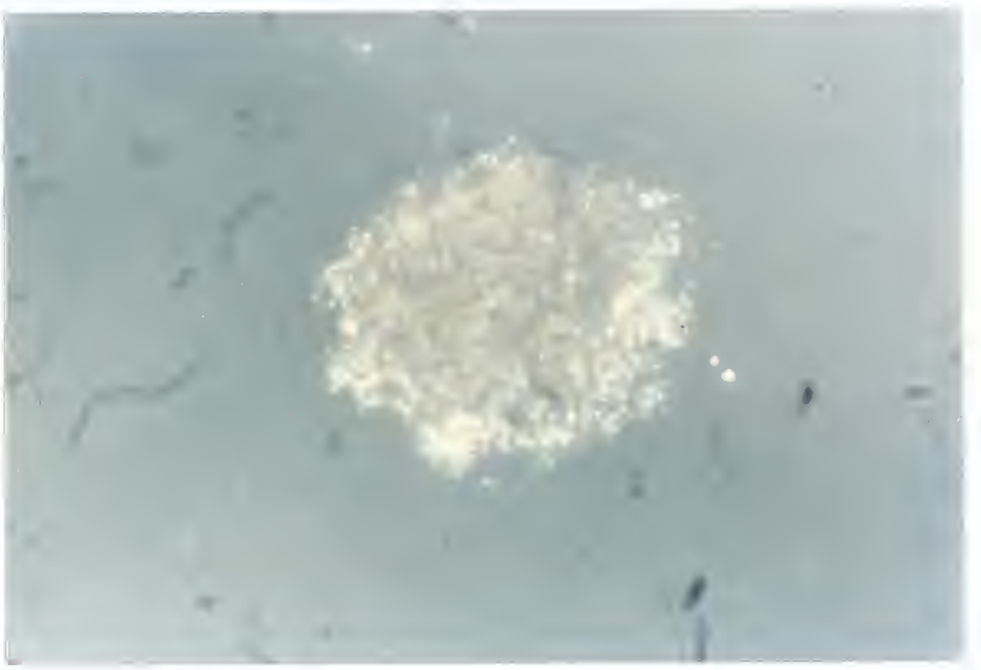


Figure 8

In this case there is no difficulty distinguishing the cell-associated grains from any background. However, the ideal conditions of incubation, immunoglobulin concentration, emulsion thickness and washing technique occurred apparently only once during the limited time available for the work. Usually we were looking at a maximum of 8 nucleus-associated grains and the distinction from background was more difficult. An example of a nucleus with asmaller number of grains is in Figure 9; here the grains are concentrated at one pole of the nucleus (Giemsa stain).

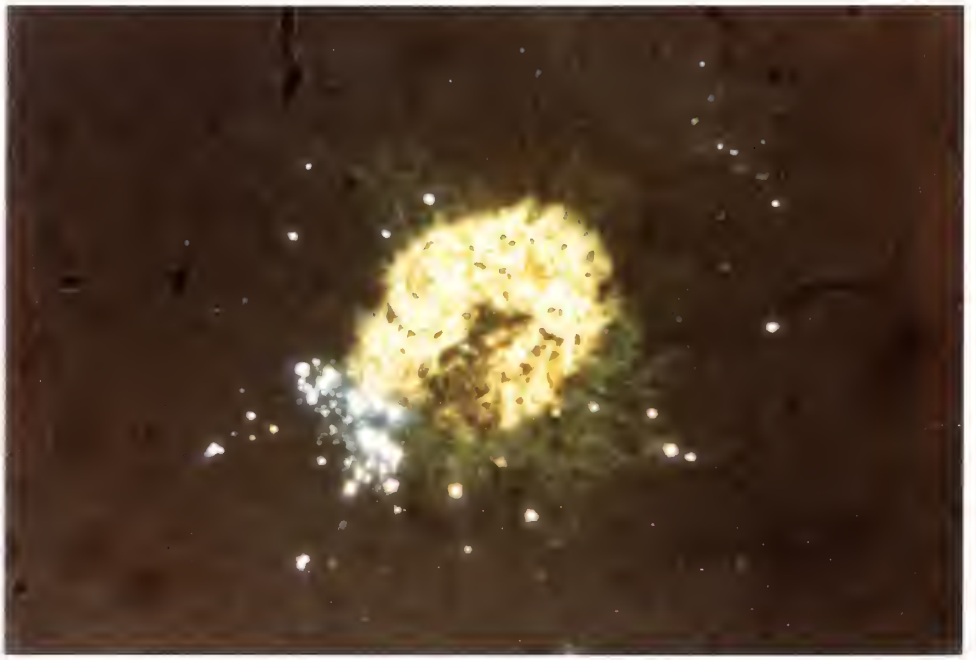


Figure 9

Figures 10 and 11 show in black and white the appearance of cells that were typically read for DNA content and associated grains. The right lower cell in Figure 10 has numerous (more than 8) grains, located at the "upper" pole:

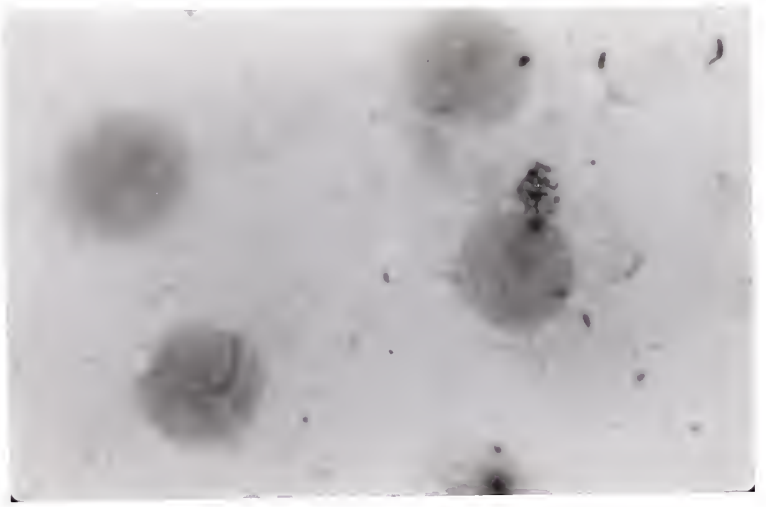


Figure 10

The cell on the right-had side of Figure 11 has two grains,

one at 12 o'clock and one at 3 o'clock:

Figure 11

It was easier to distinguish grains from dust particles under the microscope than it is in the photographs.

As one can tell from these photographs, when grains are visible at some distance from the nucleus it is impossible to tell for sure whether they are associated with the cell to which the nucleus belongs: the cytoplasm of each cell has been hydrolyzed by Feulgen staining, and the area between nuclei is shared potentially by several cells. This is the reason for our having considered grains as "cell-associated" only if they were within one diameter of the nucleus being measured.

Binding vs. DNA Content:

In the initial experiment using cytofluorimetry we found a bimodal distribution of fluorescence in the randomly-selected population, with peaks at approximately 26 and 38 (See Figure 12: note that fluroescence values have only relative meaning); and two, a *clear* diference between the fluorescence distribution patterns of the labelled and the randomly-selected populations. The labelled cells tend to accumulate toward higher values of fluorescence with the suggestion of a first peak that corresponds to the second peak of the random population, and a second peak (or peaks) at approximately one-and-one-half times that value. A set of readings from another slide (Figure 13) where cells were grouped into labelled or non-labelled shows similar patterns: for the non-labelled population, one large peak (at approximately 24), and a prolonged tailing-off of that peak toward higher values, with what might be a second peak at one-and-one-half to two times the first value; and for the labelled population, a first peak at the value of the non-labelled population's second peak (approximately 40), with a labelled second (and possibly third) peak at one-and-one-half to two times its first peak value (60 to 70). The small number of cells makes it difficult to resolve these peaks any more clearly.

Figure 12.
Cell Fluorescence vs Cell Number for Labelled vs. Randomly-Selected P388D1 cells

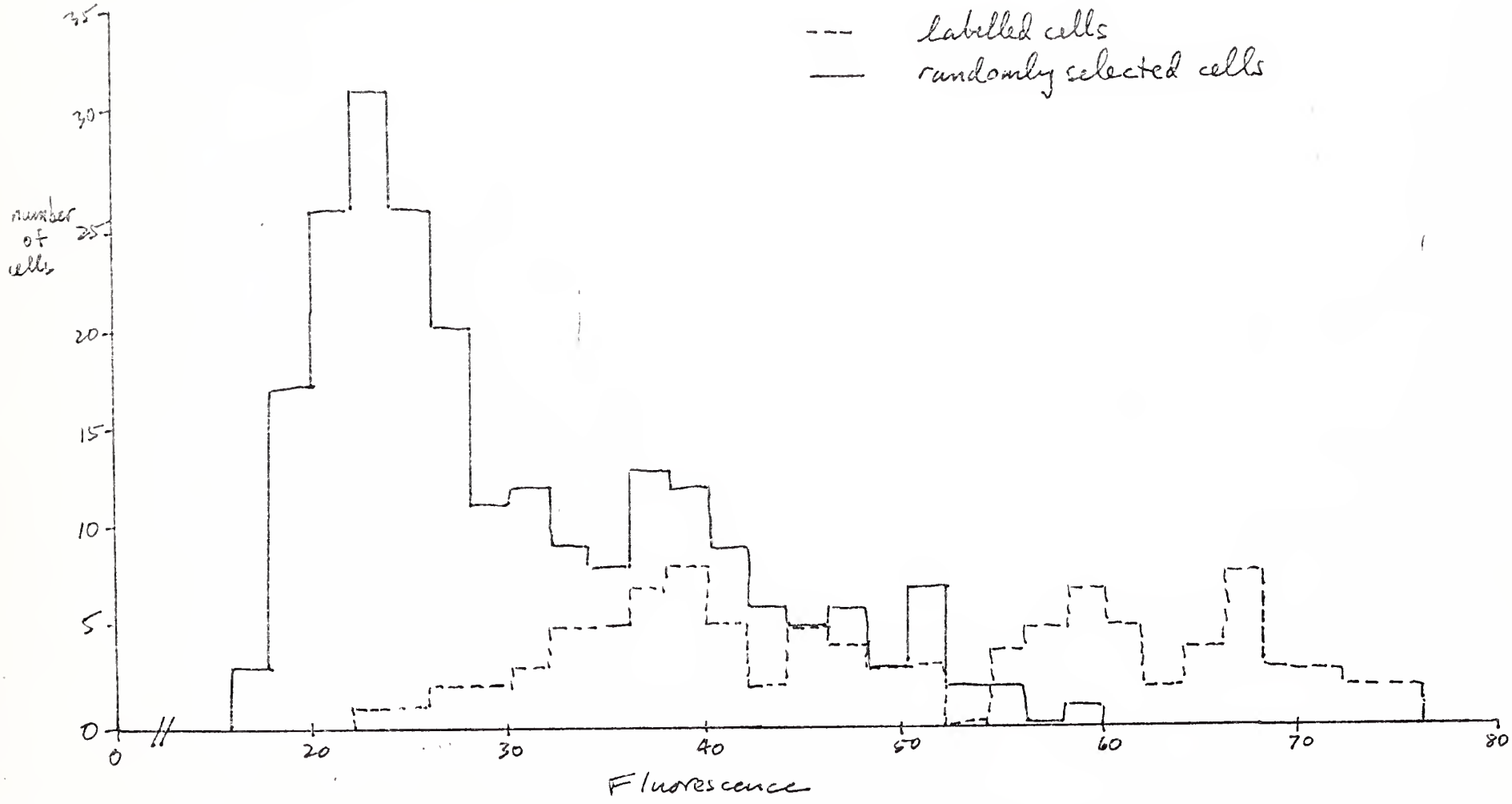
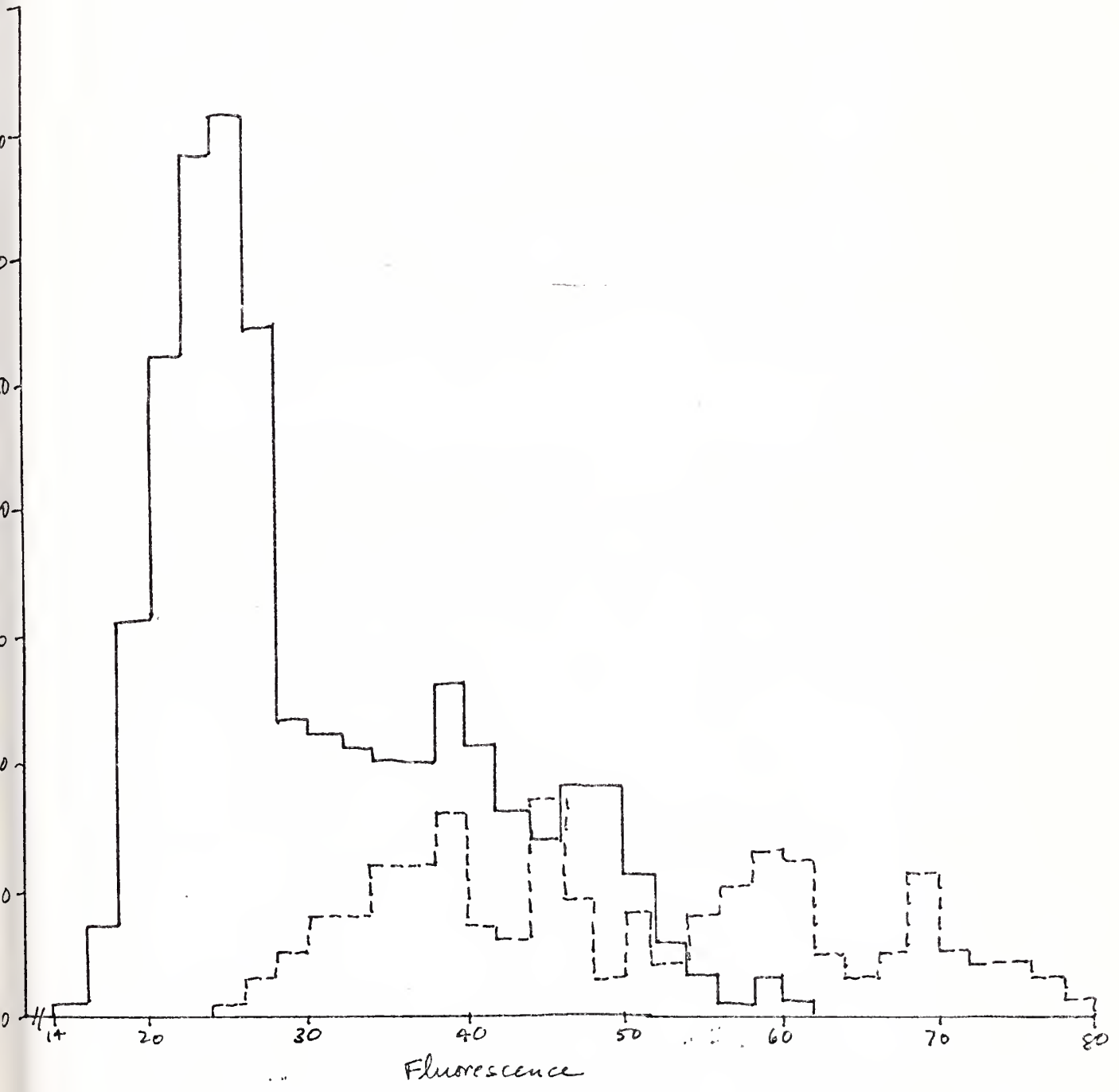


Figure 13

Cell Fluorescence vs. Cell Number for Labelled vs. Unlabelled P388D1 Cells



If one assumes that the non-labelled population is showing the expected large peak at G1 and a smaller peak at G2 (with S phase cells obscuring the dip between the two), then the labelled population has few or almost no G1 cells; it has a "G2" peak; it has a peak or peaks beyond "G2."

In the next experiment (Figure 14) a high level of background grains made it difficult to interpret the relationship of grains to cells. This forced us to group our readings crudely into cells without grains, cells with one to 20 grains, and cells with greater than 20 grains. Figure 14 shows that the "no grain," the "one to 20 grains," and the total populations have similar curves, with the expected G1-S-G2 pattern that we saw in experiment number one (Figure 12). The group with more than 20 grains, which is small, again appears to tend toward fluorescence at higher levels, with the approximate middle of its curve being at 50, compared with 40 for the G1 peak of the total population.

The background problem was worse on slides that had been incubated with a higher concentration of label; this suggested that the washing step (at this point only 30 seconds each in three volumes of cold phosphate buffered saline) needed to be lengthened and refined.

We were able to reduce background on some slides to a

Figure 14

Cell Fluorescence vs. Cell Number for Labelled and Unlabelled P388D1 Cells

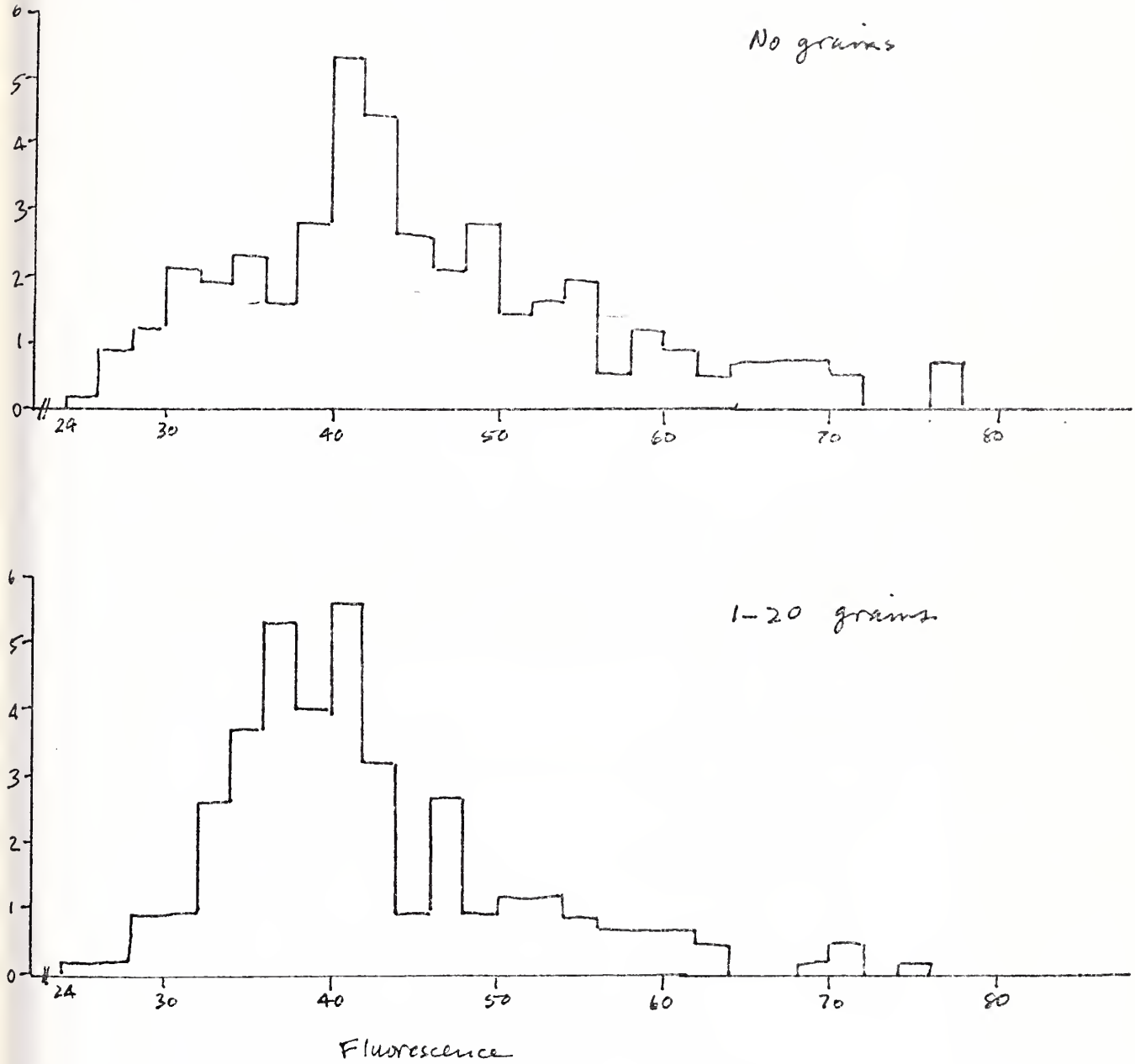
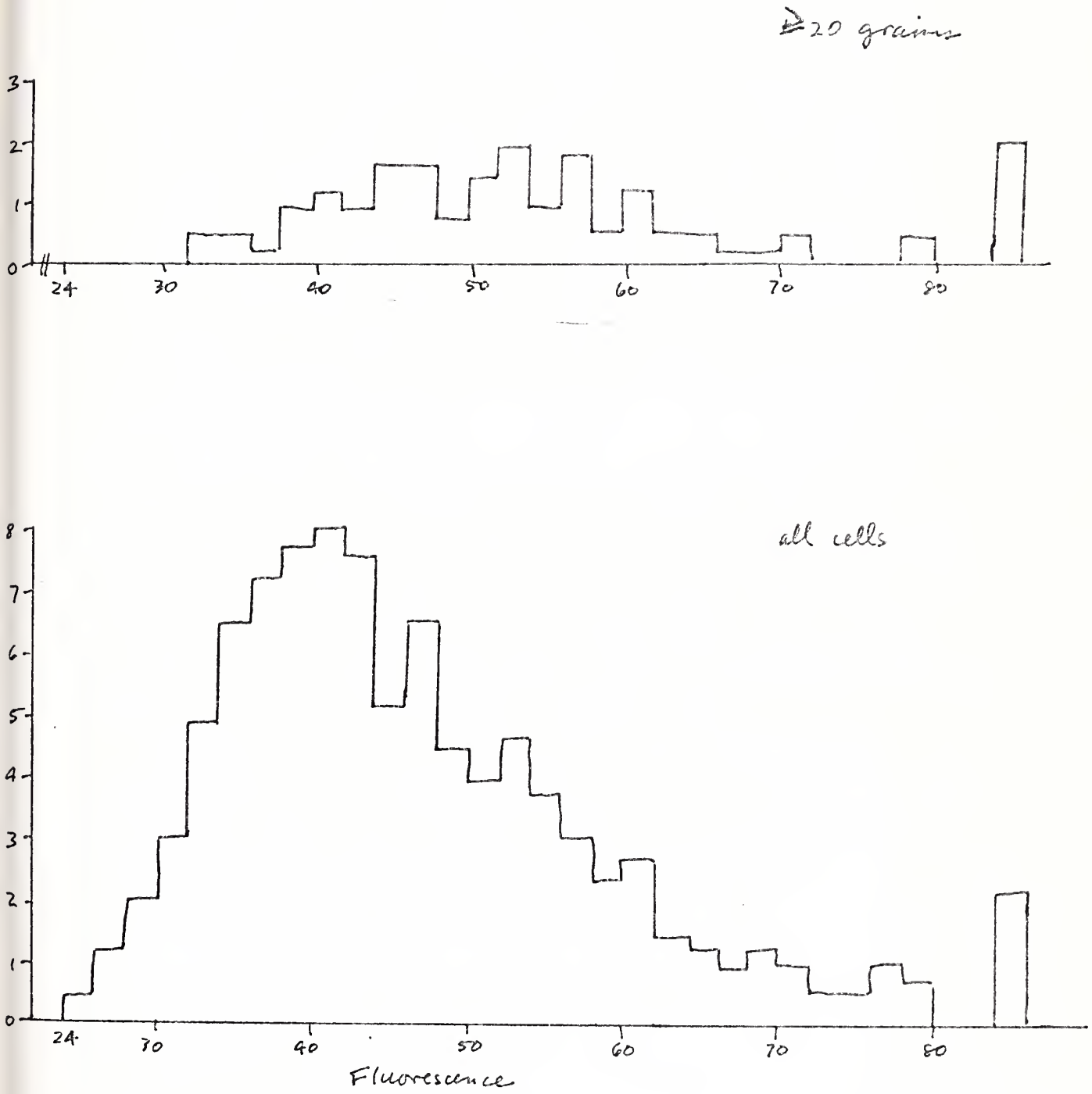


Figure 14 (cont.)



430 Cells were read at random for fluorescence and grains.

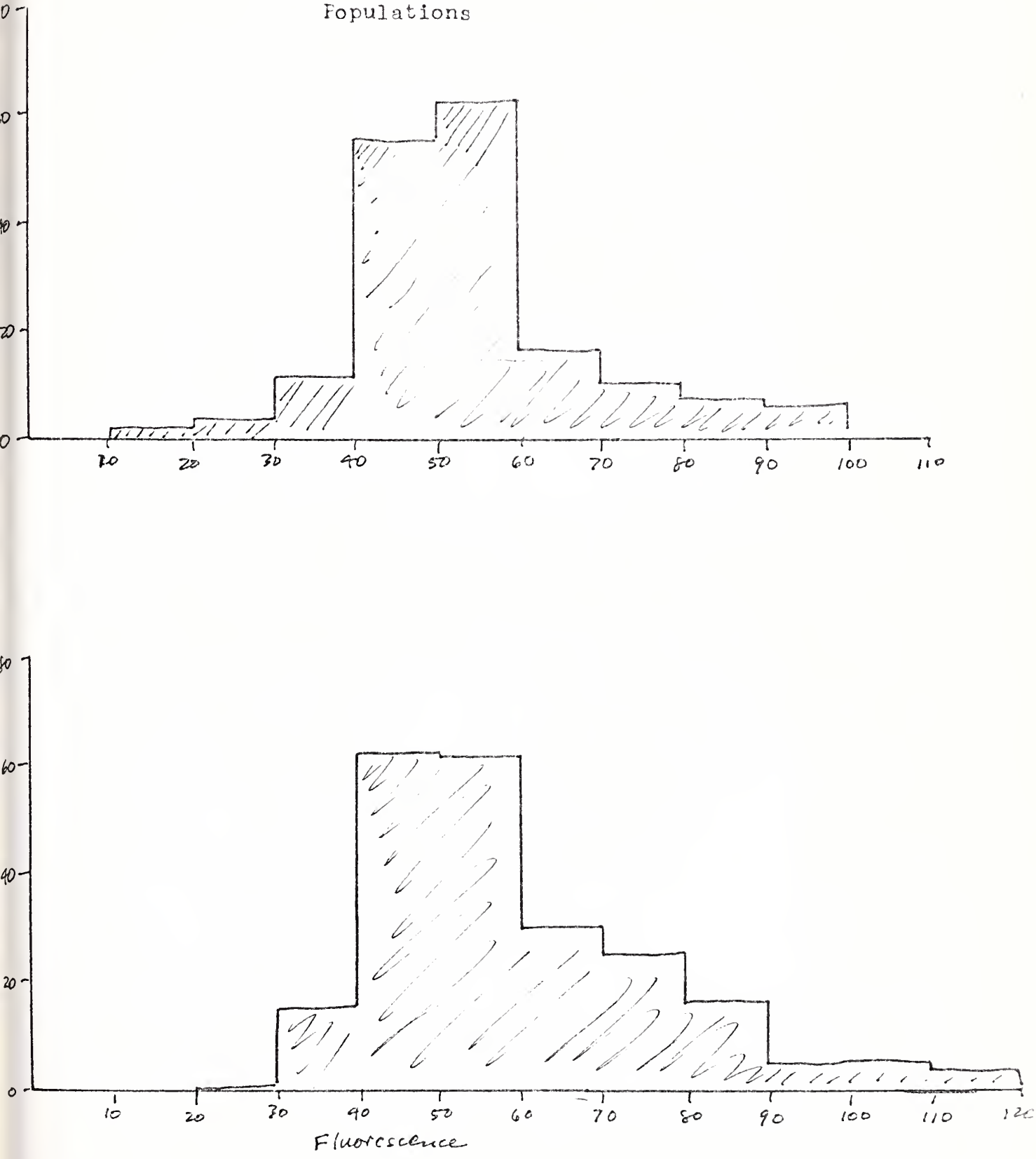
level where cell association of grains was definite and could be quantitated, although the number of grains was very small. A total of three slides was used for reading. Results were graphed as cell number vs. fluorescence for different numbers of grains after normalization (Figures 15 and 16). These graphs show a small difference in the distributions of DNA contents between the labelled cell and the unlabelled cell populations: the labelled population has an identical first peak at 40 to 60, but proportionally more of the labelled cells possess higher fluorescence values than do those in the unlabelled population. The same difference is seen whether one looks at cells with no grains vs. cells with any grains (Figure 15) or at cells with no or one grains vs. cells with two or more grains, to take account of some of the random background grain proximity to cells (Figure 16).

In these graphs one does not see the bimodal (or trimodal) peaks for labelled cells that are suggested in Figures 12 and 13. It was our observation that a much higher proportion of the cells in the later experiments (Figures 15 and 16) had grains than was the case in the earlier experiment.

A statistical analysis of the results in terms of DNA content and cell-associated grain number is shown in Table I.

Figure 15

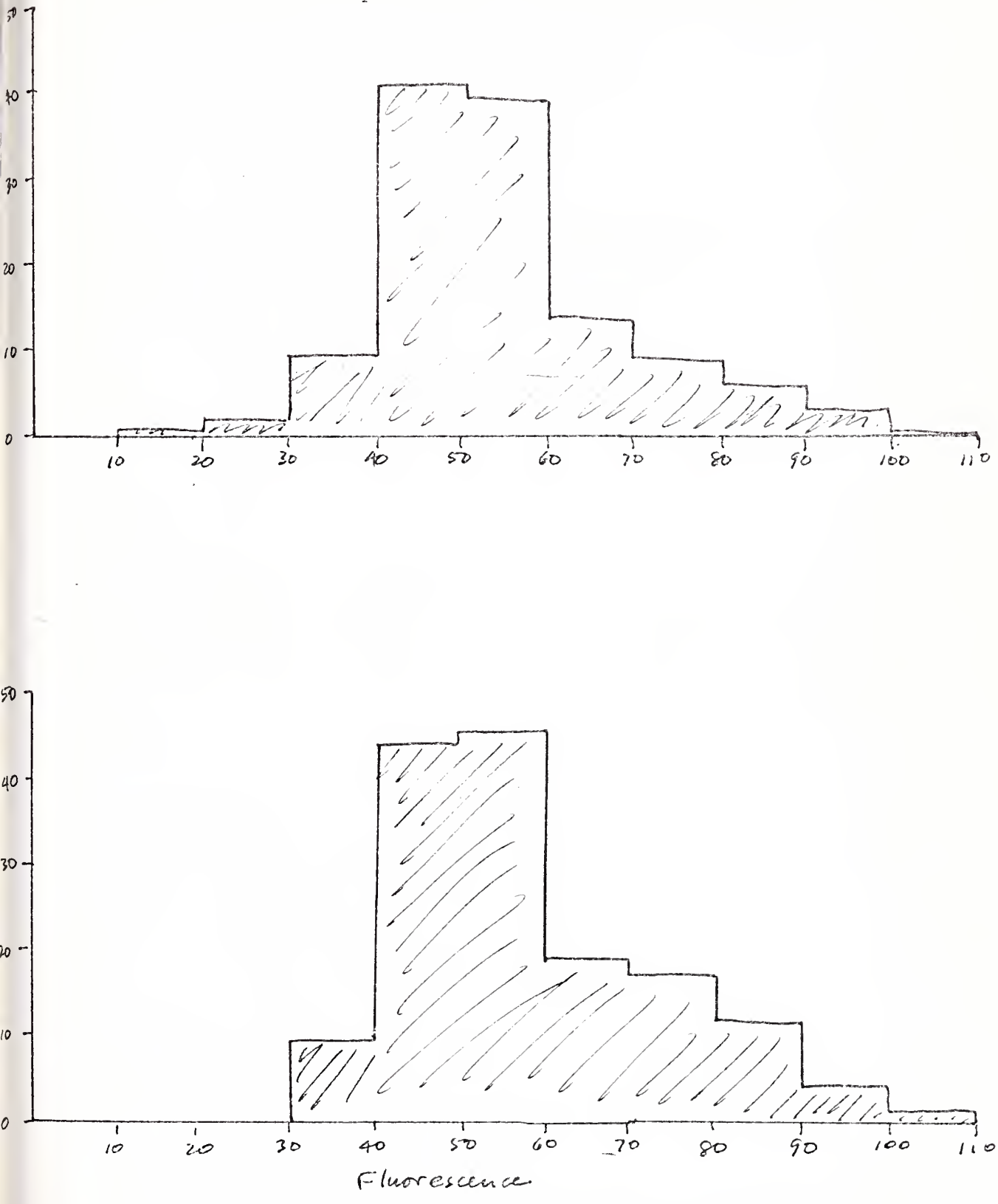
DNA Contents of Labelled and Unlabelled Cell Populations



614 Cells were read at random and results were plotted as number of cells vs. fluorescence for labelled (≥1 grains) and unlabelled (no grains) populations.

Figure 16

DNA Contents of Labelled and Unlabelled Cell Populations



614 cells were read at random and results were plotted as number of cells vs. fluorescence for labelled and unlabelled populations (unlabelled = 0+1 grains)

Using the t test for the comparison of two independent means, we determined the degree of difference in mean DNA content for populations of cells with different numbers of grains.

TABLE I

Mean DNA Content, and Significances of Differences Between the Means, for Populations of Labelled Cells

Number of Grains	Number of Cells	DNA Content (Fluorescence)	Significance of Difference From "Zero Grains" Population†
0	171	49.6 ± 14.2	------
1	108	51.5 ± 17.7	N.S.*
2	99	54.5 ± 16.6	$p < 0.01$
3	72	53.1 ± 16.1	$p < 0.10$
4	53	53.4 ± 15.9	$p < 0.10$
5	26	48.1 ± 12.3	N.S.
6	28	55.7 ± 15.3	$p < 0.05$
7	18	62.2 ± 18.0	$p < 0.001$
≥ 8	37	55.4 ± 17.1	$p < 0.05$
≥ 1	442	53.8 ± (?)	$p < 0.01$
0 + 1	279	49.7 ± 15.0	$p < 0.001$
≥ 2	334	54.4 ± 16.5	

*Not Significant (N.S.)
†Significance of differences between means was determined by using two-tailed t test for comparison of independent means.

There is a significantly higher DNA content among cells with grains vs. those without grains; among cells with two or more grains vs. those with one or no grains; and of course among most of the individual populations possessing grains vs. those with no grains.

A second type of analysis was designed to tell whether the mean number of grains changed with increases in the DNA content, which was the main question of this research project. Unfortunately, although the statistics showed a significant difference, the low number of grains per cell which were involved rendered the figures unconvincing. Therefore we can say only that there is a suggestion of an increased amount of binding with increased DNA content.

Pattern of Binding:

Photographs of Giemsa-stained cells under fluorescence revealed a nonrandom pattern of grain distribution. It is localized, rather than being diffuse over the nucleus. In Figures 7 and 8 we saw a circumferential distribution. This is found also in Figure 17. (The pale halo around the nucleus in some figures is an artifact of the microscopy.) In Figure 17 there is also a blue line underneath the grains. That this is not an optical artifact or a reflection of the fluorescent grains themselves is

demonstrated by Figures 17-21:

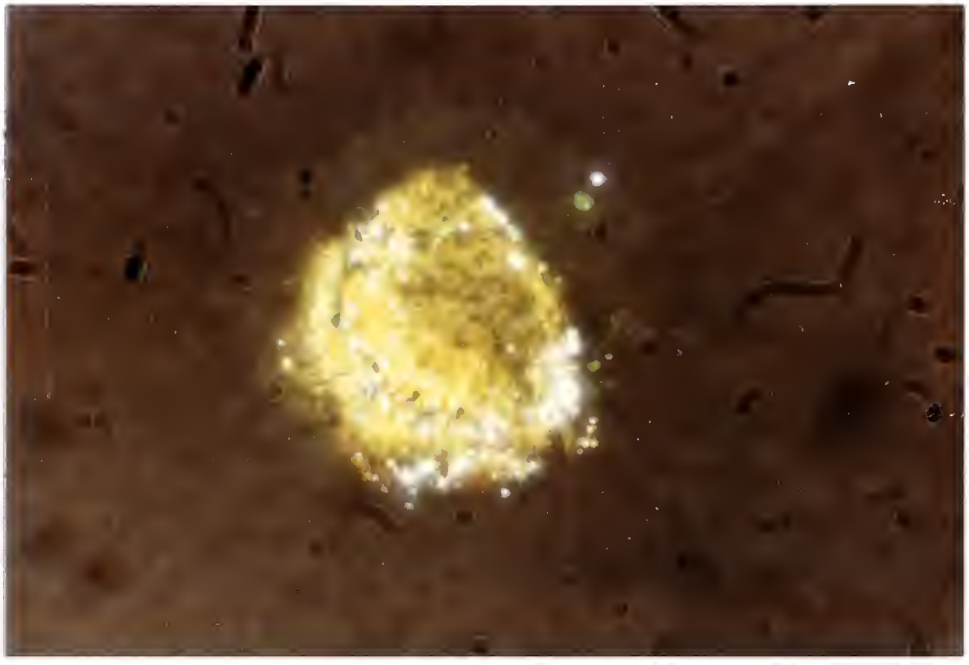


Figure 17

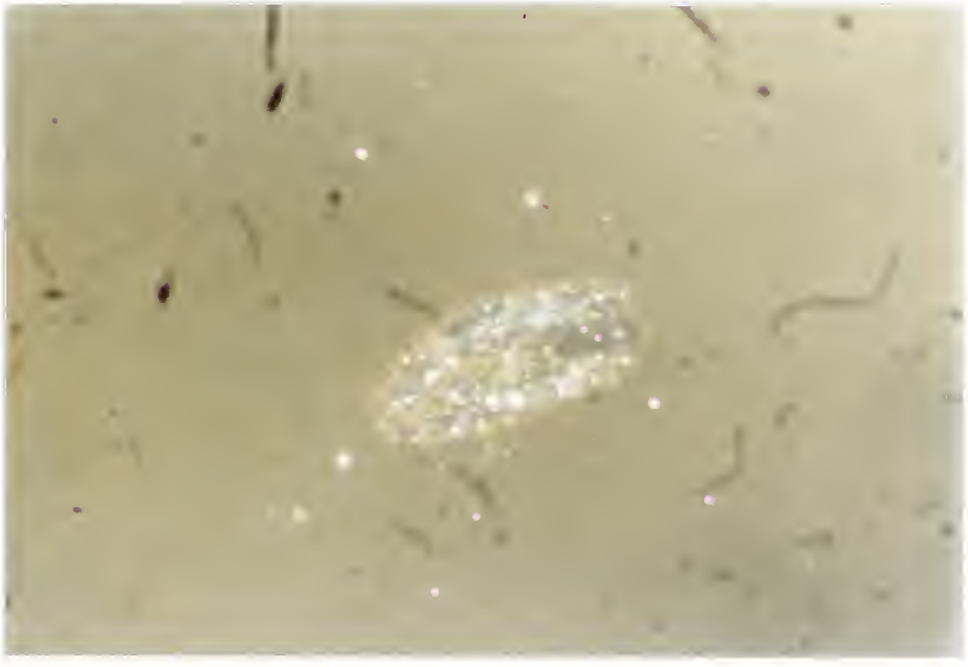


Figure 18

Figure 19

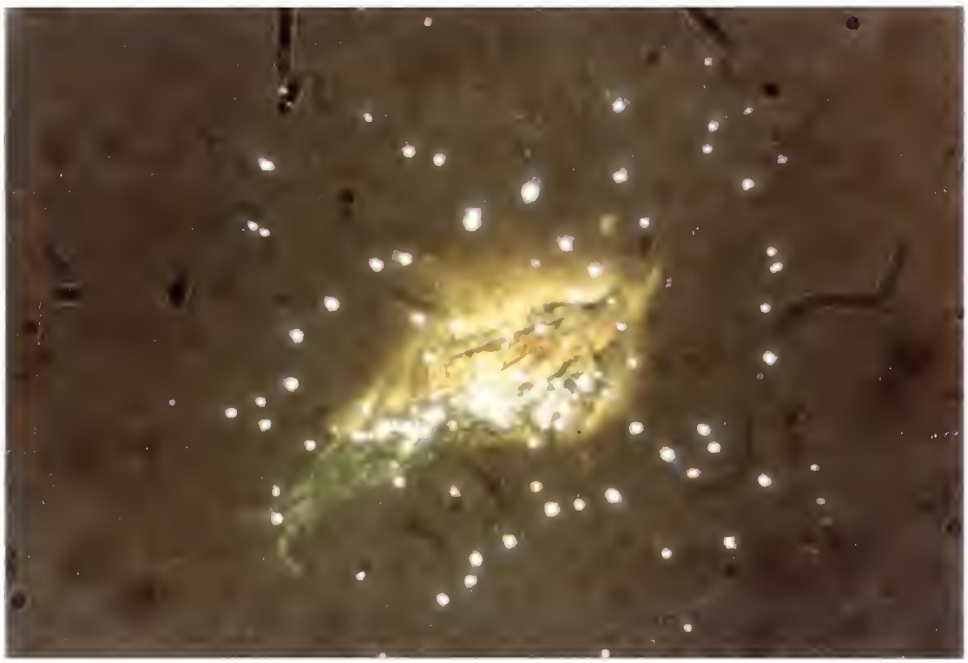


Figure 20

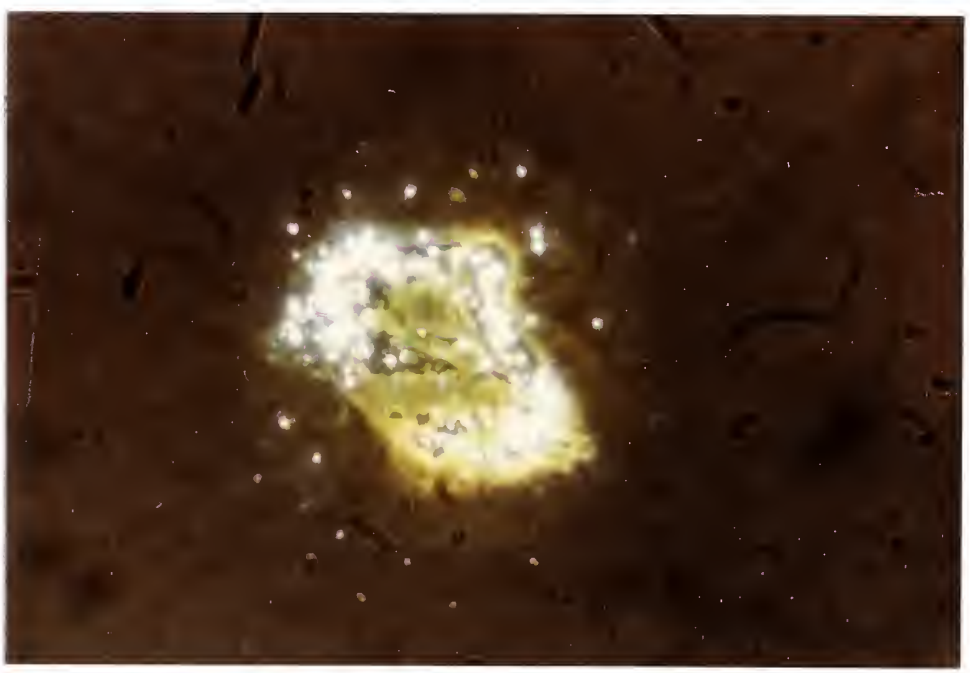


Figure 21

There is an association between the blue fluorescence and the binding sites. We do not have an explanation for this association, nor for the blue fluorescence.

Discussion

In the experiments on binding kinetics, whose purpose was to establish a basis for the later work on binding vs. cell cycle, we showed that our materials and procedures allowed us to duplicate the binding curves and constants that are in the literature on the IgG-Fc receptor interaction. We obtained consistent results if we cleared P388D1 cells of surface immunoglobulins, washed them, and incubated flasks of moderate density at 4°C for approximately one hour with(monomeric)UPC-10 IgG (mouse myeloma protein) at a concentration of approximately 10^{-7}M.

We found that the binding of labelled immunoglobulin was suppressible by unlabelled immunoglobulin, suggesting that the iodination did not destroy the immunoglobulin molecule's binding properties; we plotted a binding curve whose reciprocal plot appeared linear, suggesting a first-order binding kinetics; we calculated a K_D of 5.2×10^6 M^{-1}; and we calculated a density of approximately 3×10^5 binding sites per cell. These results agree well with those of Unkeless and Eisen (56), who investigated the binding of

several IgG2a proteins to P388D1 cells and found that the binding reaction followed pseudo-first order kinetics, with maximum binding at 4°C and with K_A values of 1.1×10^8 M^{-1} (for an IgG2a protein of a different specificity) and 7.5×10^6 M^{-1} (for an IgG2b protein). Segal and Hurwitz[42] found that UPC-10 bound to P388D1 with a K_A of from 2.3 to 8.1×10^6 M^{-1}. Their results -- all arrived at from extrapolations on best-fit Scatchard plots -- correspond well with ours. Likewise, our calculation of number of sites per cell agrees with their estimates, which are all on the order of 10^5.

We did not test the binding of either Fc fragments or other immunoglobulins to our cells, nor did we attempt to show competition for the UPC-10 binding by Fc fragments. Unkeless and Eisen have already shown that (a) two IgG2a proteins which differed considerably in their isoelectric points (presumably because of differences in their variable regions) were bound with the same affinity, and (b) with one of these proteins the Fc fragment was bound and the Fab fragment was not; these results give evidence that it is the constant region of the immunoglobulin molecule which binds to the surface receptor. The virtual identity of our binding parameters with those of Unkeless and Eisen strongly suggests that we also are dealing with binding of the Fc portion of the immunoglobulin molecule.

Our synchronization experiments showed, through spectrophotometric profiles of DNA content, progression of the cell populations through the cell cycle after release from Thymidine block. The subsequent experiment, on binding vs. DNA content with Thymidine-blocked cells, suggested some increase in binding associated with either the decrease in the G1 or the increase in the G2 populations (or both).

Several questions present themselves with respect to these admittedly tentative results. One, did the Thymidine block affect the binding? Thymidine block is a metabolic interference, and is known to have other effects on cells than simply arrest of cell cycle progress[7]. Although there is no particular reason to think that the production or unmasking of surface receptors is affected by such a procedure, there is certainly no reason why it could not be so. It is therefore at least problematical to investigate a hypothetically cell cycle-associated phenomenon through the use of a cell cycle-interfering chemical. Second, the Thymidine block is a rather crude device to investigate the cell cycle for another reason: initial synchronization is not completely successful, and the percentage of cells which remain synchronized decreases rapidly with time. Not only does this population phenomenon blur the results, it also prevents us from making possibly important distinctions between individual cells which adhere to the block

and those which do not: by looking at a whole population we miss certain differences that turn out to be associated with binding. The third question has to do with the meaning of the result: is the increase in binding truly a cell cycle-related phenomenon, or is it the result of a non-specific increase in membrane proteins correlated with the increase in surface area during cell growth?

It was the purpose of the autoradiography-cytofluorimetry experiments to look at binding vs. DNA content in a system where metabolic interference was absent and cell cycle stage could be more accurately determined. These experiments suggested, again tentatively, that in mouse macrophage-like cells of the P388D1 line, as the DNA content increases there is an increase in the amount of binding of ^{125}I-labelled immunoglobulin to the cell surface. Our hypothesis is that the display of Fc receptors on the cell surface of an immunologically significant cell is related to the cell cycle. What do the experiments permit us to say about this, and what further investigations do they suggest?

A number of questions can be raised about drawing the hypothetical conclusion from this set of experimental data. The obvious first one is that the data are suggestive but not evidential. The Giemsa-stained slide's high levels of cell-associated grains, which are visible in Figures 7

and 18-21, were not reproduced on the slides from which we took our data, which are seen in Figures 10 and 11. Time limitations prevented the further work that this problem demanded. The low numbers of grains prevented us from quantitating our results meaningfully. Since our first experiments did show that our labelled immunoglobulin bound to the cell surface receptors with the same kinetics that other investigators have found, it is likely that the problem of low numbers of grains was caused by such things as imperfect emulsion coating, inadequate time of exposure of the emulsion, difficulties with the washing and less than optimal immunoglobulin concentration for differentiation of background from cell-associated grains. It may also be that the data which we collected would turn out to correspond well with data collected on cells with more grains -- that the difference is only one of proportion. However, the only way to know this would be to obtain the more complete data.

Another objection is that we did not specifically show, through competitive inhibition by Fc fragments themselves, that we were binding our immunoglobulin to Fc receptors rather than to some other surface antigen to which, for example, the variable region of UPC-10 might have an affinity. Our evidence on this point lies in the

near-identity of our binding curves and dissociation constants with those of Unkeless and Eisen[56], who used P388D1 and UPC-10 and demonstrated binding to the Fc receptor by the inhibition experiment with Fc fragment. A more serious objection may be made to the technical problems of the grain-reading process: areas of only a few slides fulfilled the stated criteria for reading cell-associated grains, usually because of high background caused either by excess emulsion on the coverslip or by some extraneous exposure of grains to radiation (inadequate washing, for instance). Although we achieved consistent results from slide to slide for distribution of grains vs. absorbance, the exact balance of labelled vs. unlabelled immunoglobulin concentrations, washing technique, emulsion coating, and exposure time apparently occurred infrequently.

Third, are there basic questions about interpretation of the results? For example, this cell line is normally tetraploid, and there is no way in this experiment to distinguish between a tetraploid G2 cell and an octoploid G1 cell. Our finding of an occasional cell in very high absorbance ranges (we tried to avoid measuring overlapping nuclei) suggests that part of the population does have a chromosome content greater than tetraploidy. Hence, we cannot be sure whether the higher absorbance peaks for labelled cells reflect a phenomenon related to the G1-S-G2

cell cycle of the "normal" P388D1 population or whether it was a consequence of polyploidization.

Another question of interpretation is whether we can distinguish here between increased binding as a cell cycle-related (and possibly -regulated) phenomenon and increased binding as a result of the nonspecific increase in membrane proteins that occurs continually with expansion during cell growth. If one could show, for example, a sharp increase in binding at the G1/S or the S/G2 border, as opposed to a gradual and linear increase in binding with absorbance, then it would strongly suggest a process other than a nonspecific increase in membrane components during cell growth. To begin with, neither Figures 15 and 16 nor Table I enables us to make this statement. The same kind of problem was addressed by Isersky et al.[18] in their research on cell cycle-associated changes in IgE binding on rat basophilic leukemia cells: they used Coulter Counter measurement of volumes, on their populations of cells that had been separated previously by centrifugation, to establish that the display of surface receptors for IgE increased during G1 even though cell volume remained constant or else decreased. They also used scanning electron micrography to determine that cells in cultures with differing distributions of DNA content had similar

numbers of folds and microvilli, thereby showing that the changes in the numbers of receptors were not due to morphological surface changes. Reviewing some of the literature on the relationship between the density of surface markers (such as H-2 antigens) and cell volume, they concluded that direct, inverse, and independent relationships have all been found; that for their own cell line a relatively constant but nonlinear relationship held; and that a more precise determination of surface area would be required to prove that point.

Clearly the interpretation of the relationship between surface area and volume in macrophage-like cells, which spread out and elongate on the surfaces where they grow, must also be complex. This emphasizes the need to use more than one measure of cell growth in looking at potentially cell cycle-associated phenomena. What one would like to have is a technique for measuring surface area; if that were available, then a population of cells might be synchronized and grown, the surface area of members of the population measured, and the average volume determined. These parameters for populations at different stages of the cell cycle could then be correlated with immunoglobulin binding.

This research project is not finished. To complete

the work on cell cycle *vs.* binding, it is necessary, as was said above, to collect data from cells with more grains. In order to pursue the work further, the following would also seem to be necessary: (1) use of a technique for sorting into cell cycle phases without metabolic interference: (2) perfection of a reliable method for measuring the display of Fc receptors without variations in background, amount of binding and amount of grain exposure; (3) confirmation of the ability of monomeric, unlabelled immunoglobulin and of Fc fragments to inhibit completely the binding of labelled immunoglobulin to surface receptors; (4) measurement of cell volume; (5) if possible, determination of cell surface area; (6) use of more than one system for determining binding *vs.* cell cycle stage. One might construct such an experiment as follows:

1. Repeat the experiments above on binding *vs.* concentration of labelled and unlabelled immunoglobulin, demonstrating a saturation level with labelled immunoglobulin and an ability of the unlabelled material to suppress binding completely.

2. Using the technique of separating cells by centrifugation[77], obtain G1, S, and G2 populations of cells, and treat each fraction in two ways: label one with immunglobulin and measure binding (by any of the several

methods available); and analyze the other for cell volume and (if possible) surface area. If enough cells could be obtained, it would be desirable to have even a third treatment: to allow part of each fraction to grow (the cells would have become synchronized by virtue of starting at the same place in the cell cycle) before performing the same experiments as above; this would establish that the growth patterns and binding parameters have not been altered by the centrifugation procedure.

3. Label cells with fluorescein-tagged immunoglobulin and measure binding vs. cell volume vs. DNA content in a flow microfluorimeter. If this technique is in fact available it could provide the most direct test of the hypothesis that binding changes with progression through the cell cycle.

Our discussion has been focusing on only "monomeric" Fc receptor binding. Since it appears that there are at least two distinct Fc receptors, one would like to know the relationship between "aggregated" Fc receptors and the cell cycle, as well as binding patterns (see below). This would necessitate an entirely separate set of experiments along the same lines but using multimeric ligands.

Another result of our work was to demonstrate that binding of immunoglobulin to the surface membrane of P388D1 is nonrandom and may be associated with a specific cell area that appears blue under our conditions of fluorescent

lighting. Exactly what this blue area is we do not know, but the finding of nonrandom topography of binding, as we have already mentioned, recalls similar reports of nonrandom binding by other investigators[8,19,27] and has possible implications for the question of how the macrophage "decides" which immunoglobulin molecules to bind. One might follow up this finding by (1) electron microscopy of the phenomenon, to identify structures associated with Fc receptors. Radioactively labelled areas can be identified by electron microscopy and ^{125}I-labelled immunoglobulin might again be used. (2) investigation of binding patterns at different times in the cell cycle (as was done by Garrido[19]); (3) investigation of the binding pattern with labelled multimeric immunoglobulins of different sizes, for example to look for capping. (Capping, and the consequent stearic interferences of large molecules, were suggested by Knutson et al.[29] as an explanation for why the maximum number of aggregated IgG molecules per cell decreased as aggregate size increased.) Oliver and Berlin[40] hypothesize that the binding of sensitized red blood cells requires movement of Fc receptors from a random distribution into aggregates on the macrophage surface to form multiple points of contact with the erythrocyte. Such movements of Fc receptors are quite

plausible in view of the other known translational and directed intramembrane movements of other proteins and lipids during phagocytosis[40]. Functional control of the Fc receptors by the cell most likely involves both number and topography.

A final area to be investigated is the phagocytic activity of macrophages and how it relates to the cell cycle and to Fc receptor display. We attempted to measure phagocytic activity at different times in the cell cycle, using Thymidine block-synchronized cells and radioactively labelled particles of oil; however our results were inconclusive because of large ranges of error. Walker[60,66] has already found an inverse relationship between the density of Fc receptors displayed and phagocytic activity. However, these results came from separate experiments, and should be repeated. An additional complication is that phagocytic activity may correlate differently with "monomeric" than with "aggregated" Fc receptor display, and the design of experiments would have to take this into account.

Ultimately, one would like to be able to understand the relationship between the cell cycle, the density and pattern of display of different Fc receptors, and those

immunological functions of the macrophage that appear to depend at least partly on the Fc receptor, namely phagocytosis and antigen presentation. The outcome of such research would add a piece to the increasingly detailed picture of how the developing organism and the environment interact through a boundary that is at once controlled and responsive.

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